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<b>(21) International Application Number:</b> PCT/IL00/00102 <b>(22) International Filing Date:</b> 18 February 2000 (18.02.00)  <b>(30) Priority Data:</b> <table border="0"><tr><td>128587</td><td>18 February 1999 (18.02.99)</td><td>IL</td></tr><tr><td>129439</td><td>14 April 1999 (14.04.99)</td><td>IL</td></tr><tr><td>131363</td><td>11 August 1999 (11.08.99)</td><td>IL</td></tr></table> <b>(71) Applicant (for all designated States except US):</b> COMPUGEN LTD. [IL/IL]; Pinchas Rozen Street 72, 69512 Tel Aviv (IL).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> SAVITZKY, Kinneret [IL/IL]; Metodela Street 44, 69548 Tel Aviv (IL). MINTZ, Liat [IL/IL]; Harav Kook Street 14, 47254 Ramat Hasharon (IL). DAVID, Anat [IL/IL]; Afner Street 11, 53292 Givataim (IL). AZAR, Idit [IL/IL]; Hankin Street 2, 76354 Rehovot (IL).  <b>(74) Agent:</b> REINHOLD COHN AND PARTNERS; P.O.Box 4060, 61040 Tel-Aviv (IL).		128587	18 February 1999 (18.02.99)	IL	129439	14 April 1999 (14.04.99)	IL	131363	11 August 1999 (11.08.99)	IL	<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
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<b>(54) Title:</b> NOVEL NUCLEIC ACID AND AMINO ACID SEQUENCES  <b>(57) Abstract</b>  The invention concerns novel nucleic acid sequences, amino acid sequences coded thereby and method of detection using the above. The novel nucleic acid sequences are naturally occurring splice variants of a prostate specific antigen sequence (PSA) or of the KLK-2 gene.											

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## NOVEL NUCLEIC ACID AND AMINO ACID SEQUENCES

### FIELD OF THE INVENTION

The present invention concerns novel nucleic acid sequences, vectors and host cells containing them, amino acid sequences encoded by said sequences, and antibodies reactive with said amino acid sequences, as well as pharmaceutical  
5 compositions comprising any of the above. The present invention further concerns methods for screening for candidate activator or deactivators utilizing said amino acid sequences.

### BACKGROUND OF THE INVENTION

10 Prostate-specific antigen (PSA) is the most important tumor marker for early detection, staging, and monitoring of men with prostate cancer today. PSA testing has appreciable false-positive and false-negative results, particularly in the 2.5-10 ng/ml range. Measurement of the percentage of non-protein-bound (i.e. free) PSA in serum, which is lower in patients with prostate cancer, have been evaluated as a  
15 method for increasing the accuracy of PSA testing.

Thus measurement of PSA in serum, has been postulated as having potential clinical utility for increasing the sensitivity and specificity of PSA testing. Cutoff figures are affected by total PSA levels at prostate value. The prevalence rate of cancer in the screened population, depending on age, race, previous biopsy history  
20 etc., also influences the screening cutoffs. It has also been postulated that the percentage of free PSA may also correlate with a potential aggressiveness of early-stage prostate cancer. Thus, the level of free PSA may not only be used in order to diagnose prostate cancer, but also to predict the course of development of this cancer, and the patient's prognosis, and decide on a suitable treatment regime.

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Human kallikrein-2 gene (termed herein after: "KLK" which is also known as KLK-2) is transcribed from the same locus as the PSA and is also known to be prostate specific. It has been speculated that both PSA and KLK have common expression control such as common enhancer and/or promoter and both function as  
5 serine proteases.

## GLOSSARY

In the following description and claims use will be made, at times, with a variety of terms, and the meaning of such terms as they should be construed in  
10 accordance with the invention is as follows:

**"Prostate specific antigen (PSA) variant"** – the sequence shown in any one of SEQ ID NO: 1 to SEQ ID NO: 6, sequences having at least 70% identity to said sequence and fragments of the above sequences of least 20 b.p. long. SEQ ID NO:  
15 1 to ID NO:5 are nucleic acid sequences which resulted from alternative splicing of the native and known PSA sequence appearing in HSPSAR and HUMPSANTIG (GenBank Acc. X05332 and M24543, respectively). It should be emphasized that the PSA variants of the invention are naturally occurring sequences resulting from the alternative splicing of the RNA transcribed from the PSA gene and not merely  
20 truncated or mutated forms of the gene. SEQ ID NO: 6 is an alternative splice variant of the human kallikrein-2 gene (KLK-2) appearing in GenBank as KLK2 under Accession Number NM\_005551.

SEQ ID NO: 1 – (PSAL\_0): The nucleic acid sequence starting in position 4364 of the HUMPSANTIG up to position 7305, then a different sequence. The coded  
25 peptide (SEQ ID NO:7) starting identically to the original PSA for 16 aa, then a different sequence which is transcribed from the PSA intron between exons 1 and 2.

SEQ ID NO:2 – (PSAL\_1):

Nucleic acid sequence identical to SEQ 1.

Peptide (SEQ ID NO: 8) – Starting in a Methionine 114 aa upstream from the  
30 original PSA, and has the same 16 aa identity and 3' end as PSAL\_0.

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SEQ ID NO:3 – (PSAL\_2):

Nucleic acid sequence which starts in same place as PSAL\_0 but goes up to position 6336 of the HUMPSANTIG, then continues in a different sequence.

Peptide (SEQ ID NO: 9) – Identical to PSAL\_1 peptide.

5 SEQ ID NO: 4 – (PSAL\_5):

Nucleic acid sequence which starts in same place as PSAL\_0, goes up to position 6069 of HUMPSANTIG and end there (original intron).

Peptide (SEQ ID NO:10) - Has same starting place as PSAL\_1, the same 16 aa identity to PSA, then a different intron region translated.

10 SEQ ID NO: 5 – ( PSAL\_6):

Nucleic acid sequence starts in the same place as PSAL\_0, goes up to position 5913 of HUMPSANTIG, then enter the original PSA exon # 2 and continues.

Peptide (SEQ ID NO:11) has same starting place as PSAL\_1, then enters the same identity region and continues as the original PSA until the end.

15 SEQ ID NO:6 is a splice variant of the KLK-2 that includes coding region from the original KLK-2 intron between exons 1 and 2. The term of “*PSA variant*” in the context of the present invention concerns splice variants of the known PSA gene as well as splice variants of the KLK-2 gene, which is also known to code for antigens specific to the prostate.

20

***“Prostate specific antigen variant product (PSA variant product) – also referred at times as the “PSA variant protein” or “PSA variant polypeptide” - an amino acid sequence coded by said PSA variant nucleic acid sequence. The amino acid sequence may be a peptide, a protein, as well as peptides or proteins having***  
25 ***chemically modified*** amino acids (see below) such as a glycopeptide or glycoprotein. An example of a PSA variant product is shown in any one of SEQ ID NO: 7 to SEQ ID NO: 12, and includes also analogues of said sequences in which one or more amino acids has been added, deleted, *substituted* (see below) or *chemically modified* (see below) as well as fragments of this sequence having at

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least 10 amino acids. The products may be membrane associated or present in a free form in body fluids, for example in the serum.

5 *"Nucleic acid sequence"* – a sequence composed of DNA nucleotides, RNA nucleotides or a combination of both types and may includes natural nucleotides, chemically modified nucleotides and synthetic nucleotides.

10 *"Amino acid sequence"* – a sequence composed of any one of the 20 naturally appearing amino acids, amino acids which have been *chemically modified* (see below), or composed of synthetic amino acids.

*"Fragment of PSA variant product"* - a sequence which is the same as part of but not all of the amino acid sequence of the PSA variant product.

15 *"Fragments of PSA variant nucleic acid sequence"* a continuous portion, preferably of about 20 nucleic acid sequences of the PSA variant nucleic acid sequence (see below), which sequence does not appear in the original PSA.

20 *"Conservative substitution"* - refers to the substitution of an amino acid in one class by an amino acid of the same class, where a class is defined by common physicochemical amino acid side chain properties and high substitution frequencies in homologous proteins found in nature, as determined, for example, by a standard Dayhoff frequency exchange matrix or BLOSUM matrix. [Six general classes of amino acid side chains have been categorized and include:  
25 Class I (Cys); Class II (Ser, Thr, Pro, Ala, Gly); Class III (Asn, Asp, Gln, Glu); Class IV (His, Arg, Lys); Class V (Ile, Leu, Val, Met); and Class VI (Phe, Tyr, Trp). For example, substitution of an Asp for another class III residue such as Asn, Gln, or Glu, is a conservative substitution.

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**"Non-conservative substitution"** - refers to the substitution of an amino acid in one class with an amino acid from another class; for example, substitution of an Ala, a class II residue, with a class III residue such as Asp, Asn, Glu, or Gln.

- 5 **"Chemically modified"** - when referring to the product of the invention, means a product (protein) where at least one of its amino acid residues is modified either by natural processes, such as processing or other post-translational modifications, or by chemical modification techniques which are well known in the art. Among the numerous known modifications typical, but not exclusive examples include:
- 10 acetylation, acylation, amidation, ADP-ribosylation, glycosylation, GPI anchor formation, covalent attachment of a lipid or lipid derivative, methylation, myristylation, pegylation, prenylation, phosphorylation, ubiquitination, or any similar process.
- 15 **"Biologically active"** - refers to a PSA variant product which has the ability to serve as a marker of cancer, of predisposition to cancer, or of malignancy of a tumor.

**"Immunologically active"** defines the capability of a natural, recombinant or

20 synthetic PSA variant product, or any fragment thereof, to induce a specific immune response in appropriate animals or cells and to bind with specific antibodies. Thus, for example, a biologically active fragment of PSA variant product denotes a fragment which retains some or all of the biological properties of the PSA variant product, e.g the ability to serve as a marker for prostate

25 cancer; an immunologically active fragment is a fragment which can bind specific anti-PSA variant product antibodies or "*distinguishing antibodies*" (see below) which can elicit an immune response which will generate such antibodies or cause proliferation of PSA variant product-specific immune cells. The fragment will also be denoted hereinafter as "*distinguishing amino acid*

30 *sequence*".

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**"Optimal alignment"** - is defined as an alignment giving the highest percent identity score. Such alignment can be performed using a variety of commercially available sequence analysis programs, such as the local alignment program LALIGN using a ktup of 1, default parameters and the default PAM. A preferred alignment is the one performed using the CLUSTAL-W program from MacVector (TM), operated with an open gap penalty of 10.0, an extended gap penalty of 0.1, and a BLOSUM similarity matrix. If a gap needs to be inserted into a first sequence to optimally align it with a second sequence, the percent identity is calculated using only the residues that are paired with a corresponding amino acid residue (i.e., the calculation does not consider residues in the second sequences that are in the "gap" of the first sequence).

**"Having at least X% identity"** - with respect to two amino acid or nucleic acid sequence sequences, refers to the percentage of residues that are identical in the two sequences when the sequences are optimally aligned. Thus, 90% amino acid sequence identity means that 90% of the amino acids in two or more optimally aligned polypeptide sequences are identical.

**"Isolated nucleic acid molecule having an PSA variant nucleic acid sequence"** - is a nucleic acid molecule that includes the coding PSA variant nucleic acid sequences. Said isolated nucleic acid molecule may include the PSA variant nucleic acid sequence as an independent insert; may include the PSA variant nucleic acid sequence fused to an additional coding sequences, encoding together a fusion protein in which the PSA variant coding sequence is the dominant coding sequence (for example, the additional coding sequence may code for a signal peptide); the PSA variant nucleic acid sequence may be in combination with non-coding sequences, e.g., introns or control elements, such as promoter and terminator elements or 5' and/or 3' untranslated regions, effective for expression of the coding sequence in a suitable host; or may be a vector in which the PSA variant protein coding sequence is a heterologous.



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**"Expression vector"** - refers to vectors that have the ability to incorporate and express heterologous DNA fragments in a foreign cell. Many prokaryotic and eukaryotic expression vectors are known and/or commercially available. Selection of appropriate expression vectors is within the knowledge of those  
5 having skill in the art.

**"Deletion"** - is a change in either nucleotide or amino acid sequence in which one or more nucleotides or amino acid residues, respectively, are absent.

10 **"Insertion" or "addition"** - is that change in a nucleotide or amino acid sequence which has resulted in the addition of one or more nucleotides or amino acid residues, respectively, as compared to the naturally occurring sequence.

**"Substitution"** - replacement of one or more nucleotides or amino acids by  
15 different nucleotides or amino acids, respectively. As regards amino acid sequences the substitution may be conservative or non-conservative.

**"Antibody"** - refers to IgG, IgM, IgD, IgA, and IgG antibody. The definition includes polyclonal antibodies or monoclonal antibodies. This term refers to  
20 whole antibodies or fragments of the antibodies comprising the antigen-binding domain of the anti-PSA variant product antibodies, e.g. antibodies without the Fc portion, single chain antibodies, fragments consisting of essentially only the variable, antigen-binding domain of the antibody, etc.

25 **"Distinguishing antibody"** - an antibody capable of binding only to the novel PSA variant product of the invention while not binding to the original PSA product, i.e. an antibody recognizing an additional amino acid sequence which appears only in the variant product of the invention and not in the original PSA sequence. This term may also refer at times to antibodies which bind a sequence  
30 present in the original PSA and not present in the PSA variant product.

***"Distinguishing amino acid sequence"*** – an amino acid sequence of at least two amino acids which are present only in the PSA variant of the invention and not in the original PSA of which are used to prepare the above distinguishing antibodies.

5

***"Activator"*** - as used herein, refers to a molecule which mimics the effect of the natural PSA variant product or at times even increases or prolongs the duration of the biological activity of said product, as compared to that induced by the natural product. The mechanism may be by binding to the PSA variant receptor, by  
10 prolonging the lifetime of the PSA variant, by increasing the activity of the PSA variant on its target, by increasing the affinity of PSA variant to its receptor, etc. Activators may be polypeptides, nucleic acids, carbohydrates, lipids, or derivatives thereof, or any other molecules which can bind to and activate the PSA variant product.

15

***"Deactivator"*** - refers to a molecule which modulates the activity of the PSA variant product in an opposite manner to that of the activator, by decreasing or shortening the duration of the biological activity of the PSA variant product. This may be done by blocking the binding of the PSA variant to its receptor,  
20 competitive or non competitive inhibitor, by causing rapid degradation of the PSA variant, etc. Deactivators may be polypeptides, nucleic acids, carbohydrates, lipids, or derivatives thereof, or any other molecules which bind to and modulate the activity of said product.

25 ***"Treating a disease"*** - refers to administering a therapeutic substance effective to ameliorate symptoms associated with a disease, to lessen the severity or cure the disease, or to prevent the disease from occurring. In the context of the invention the disease is typically cancer and in particular prostate cancer.

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“**Detection**” – refers to a method of detection of a disease, such as prostate cancer. May be detection of an active disease or detection of a predisposition to a disease. By another alternative the detection may be capable of distinguishing between benign and malignant conditions. This term may also be used in connection with a method for evaluating the aggressiveness of a malignant state in order to correctly predict the prognosis of the patient, and in that case the detection may be used to assess the stage of the tumor.

“**Probe**” – the PSA variant nucleic acid sequence, or a sequence (including fragments) complementary therewith, when used to detect presence of other similar sequences in a sample. The detection is carried out by identification of hybridization complexes between the probe and the assayed sequence. The probe may be attached to a solid support or to a detectable label. The probe may be a fragment of any one of the SEQ ID NO: 1 to SEQ ID NO: 6 (including a fragment of the non-coding region) which is of sufficient length to hybridize to the PSA variants at a level significantly different from the binding to the original PSA sequence. The probes may also be used to detect the polymorphisms described in the nucleic acid for the purpose of determining predisposition to cancer, especially prostate cancer in healthy individuals, and for detecting loss of heterozygosity in prostate tissues as part of a malignant transformation. The probes may be used in any method of performing this assay, including primer-specific PCR, allele-specific oligonucleotide assay, restriction fragment length differences, and mini-sequencing.

“**Targeting**” – directing a compound or drug to a desired cell population. Targeting is carried out by conjugating to the compound or drug an agent capable of binding specifically to the desired cell population, while not binding to non-desired cell populations. A specific example is targeting cytotoxic drugs directed only to tumor cells, more specifically directed to prostate tumor cells, for example, by conjugating the drug to an antibody of the invention.

*"Original PSA sequence"* – the known sequence of PSA as appears in GenBank HSPSAR locus and Acc # X05332 , as well as to the known KLK-2 sequence as appears in GenBank KLK2 (NM\_005551).

5

## SUMMARY OF THE INVENTION

The present invention provides by its first aspect, a novel isolated nucleic acid molecule comprising or consisting of the coding sequence of any one of SEQ ID NO: 1 to SEQ ID NO: 6, fragments of said coding sequence having at least 20  
10 nucleic acids, or a molecule comprising a sequence having at 90% identity to any one of SEQ ID NO:1 to SEQ ID NO: 6. Preferably, the fragments should be such that they comprise sequences present in the PSA variants of the invention and not a sequence present in the original PSA (the term *"original PSA"* also includes the KLK-2 sequence).

15 These sequences are novel splice variants which results from alternative splicing of the original PSA sequence (this term according to the glossary refers also to the KLK-2sequence).

The present invention further provides a protein or polypeptide comprising or consisting of an amino acid sequence encoded by any of the above nucleic acid  
20 sequences, termed herein *"PSA variant product"*, for example, an amino acid sequence having the sequence as depicted in any one of SEQ ID NO: 7 to SEQ ID NO: 12 fragments of the above amino acid sequence having a length of at least 10 amino acids, in particular fragments comprising sequences which do not appear in the original PSA sequence, as well as homologues of the amino acid sequences  
25 SEQ ID NO.:7 to SEQ ID NO: 12 in which one or more of the amino acid residues has been substituted (by conservative or non-conservative substitution) added, deleted, or chemically modified.

The novel PSA variant products of the invention may have the same physiological activity as the original PSA peptide (this term refers also to the  
30 KLK-2 product) from which they are varied (although perhaps at a different level);

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may have an opposite physiological activity from the activity featured by the original peptide from which they are varied; may have a completely different, unrelated activity to the activity of the original from which they are varied; or alternatively may have no activity at all and this may lead to various diseases or pathological conditions.

The novel variants of the invention whether being nucleic acid or amino acid sequences may serve for detection purposes, i.e. their presence or level may be indicative of prostate cancer, predisposition to prostate cancer, malignancy of the cancer, stage of the cancer, or may be indicative to normal condition. Alternatively the ratio between the level of each variant and the level original PSA sequence from which it has been varied; the ratio of each variant to the or other variants; the total amount (sum) of two or more variants either by itself or compared to other variants; or the sum of two or more variants, may be indicative of cancer or predisposition to cancer in general, and prostate cancer or predisposition to prostate cancer in particular, as well as indicative of the malignancy of the cancer, its stage of development or of normal condition. The variants may be detected in blood or serum or in the prostate gland, the ovary, breast or salivary glands, which may share gene properties with the prostate gland. The variant products may be soluble or membrane bound.

For example, for detection purposes, it is possible to establish differential expression of the various variants in various tissues. A certain variant may be expressed mainly in one tissue, while the original PSA sequence may be expressed mainly in another tissue such as the prostate. Understanding of the distribution of the variants in various tissues may be helpful in basic research, for understanding the physiological function of the genes as well as may help in targeting pharmaceuticals or developing pharmaceuticals.

The study of the variants may also be helpful to distinguish various stages in the life cycles of the same type of cells which may also be helpful for development of pharmaceuticals for various pathological conditions in which cell cycles is un-normal, notably cancer. For example, various stages in the development of

prostate cancer may be characterized by expression, or change in level of individual PSA variants of the invention .

Thus the detection may by determination of the presence or the level of expression of the variant within a specific cell population, comprising said presence  
5 or level between various cell types in a tissue, between different tissues and between individuals.

The present invention further provides nucleic acid molecule comprising or consisting of a sequence which encodes the above amino acid sequences, (including the fragments and analogs of the amino acid sequences). Due to the  
10 degenerative nature of the genetic code, a plurality of alternative nucleic acid sequences, beyond those of SEQ ID NO:1 to SEQ ID NO: 6, can code for the amino acid sequences of the invention. Those alternative nucleic acid sequences which code for the amino acid sequences codes by any one of the sequence SEQ ID NO: 1 to SEQ ID NO: 6 are also an aspect of the of the present invention.

15 The present invention further provides expression vectors and cloning vectors comprising any of the above nucleic acid sequences, as well as host cells transfected by said vectors.

The present invention still further provides pharmaceutical compositions comprising, as an active ingredient, said nucleic acid molecules, said expression  
20 vectors, or said protein or polypeptide.

These pharmaceutical compositions are suitable for the treatment of diseases and pathological conditions, which can be ameliorated or cured by raising the level of the PSA variant product, for example for the treatment of prostate cancer, or for inhibiting the transformation from prostate hyperplasia to malignancy. By another  
25 aspect, the present invention provides a nucleic acid molecule comprising or consisting of a non-coding sequence which is complementary to that of any one of SEQ ID NO:1 to SEQ ID NO: 6, or complementary to a sequence having at least 90% identity to said sequence or a fragment of said two sequences. The complementary sequence may be a DNA sequence which hybridizes with any one  
30 of the SEQ of ID NO:1 to SEQ ID NO: 6 or hybridizes to a portion of that

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sequence having a length sufficient to inhibit the transcription of the complementary sequence. The complementary sequence may be a DNA sequence which can be transcribed into an mRNA being an antisense to the mRNA transcribed from SEQ ID NO:1 to SEQ ID NO: 6 or into an mRNA which is an antisense to a fragment of the mRNA transcribed from SEQ ID NO:1 to SEQ ID NO: 6 which has a length sufficient to hybridize with the mRNA transcribed from SEQ ID NO: 1 to SEQ ID NO:6, so as to inhibit its translation. The complementary sequence may also be the mRNA or the fragment of the mRNA itself.

10 The nucleic acids of the invention may be used for therapeutic or diagnostic applications for example for detection of the expression of PSA variant in various tissues which may be indicative to the presence of prostate cancer, indicative of pre-disposition to prostate cancer, as well as indicative of the malignancy and hence the prognosis of the prostate cancer. The variants of the invention may also be  
15 indicative of other types of cancer from glands binding physiological similarity to the prostate gland such as ovary, breast, and salivary gland.

The present invention also provides expression vectors comprising any one of the above defined complementary nucleic acid sequences and host cells transfected with said nucleic acid sequences or vectors, being complementary to  
20 those specified in the first aspect of the invention.

The invention also provides anti-PSA variant product antibodies, namely antibodies directed against the PSA variant product which specifically bind to said PSA variant product. Said antibodies are useful both for diagnostic and therapeutic purposes. For example said antibody may be used to detect the presence of prostate  
25 specific antigen-variant product in various tissues which may be indicative of the presence of prostate cancer of a predisposition for having prostate cancer, or of the malignancy of prostate cancer.

The present invention further concerns distinguishing antibodies which can bind only to a sequence present in the variants of the invention which is not present  
30 (as a continuous sequence) in the original PSA sequence. The present invention

further concerns amino acid sequences for producing said distinguishing antibodies termed "*distinguishing amino acid sequences*" which are sequences present in the novel PSA variant and not present (as a continuous sequence) in the original PSA. An example of such a sequence is the sequence of positions 33-51 in SEQ ID NO:7  
5 being:

Cys-Gln-Ala-Glu-Leu-Ser-Pro-Pro-Thr-Gln-His-Pro-Ser-Pro-Asp-Arg-Glu-Leu

The present invention also provides pharmaceutical compositions comprising, as an active ingredient, the nucleic acid molecules which comprise or consist of said complementary sequences, or of a vector comprising said  
10 complementary sequences. Alternatively, the pharmaceutical composition can comprise, as an active ingredient, said anti-PSA variant product antibodies, or said distinguishing antibodies.

The pharmaceutical compositions comprising said anti-PSA variant product antibodies, said distinguishing antibodies or the nucleic acid molecule comprising  
15 said complementary sequence, are suitable for the treatment of diseases and pathological conditions where a therapeutically beneficial effect may be achieved by neutralizing at least one of the PSA variants or decreasing the amount of the PSA variant product or blocking its binding to the receptor, for example, by the neutralizing effect of the antibodies, or by the effect of the antisense mRNA in  
20 decreasing expression level of the PSA variant product. An example of such a disease is prostate cancer. Furthermore, where the PSA variant is membrane bound, the anti-PSA variant antibodies may be used to target cytotoxic or cytostatic compounds to the tumor cells, in particular to prostate tumor cells. Since PSA variants may be produced specifically by prostate tumor cells, (and not normal  
25 prostate cells) and since this protein may be membrane associated, conjugates of anti-PSA variant antibodies and a drug can be targeted only to tumor cells and not harm healthy cells.

According to the third aspect of the invention the present invention provides methods for detecting the level of the transcript (mRNA) of said PSA variant  
30 product in a body fluid sample, or in a specific tissue sample, for example by use of



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probes comprising or consisting of said sequences (which may be a coding or uncoding sequence), as well as methods for detecting levels of expression of said product in tissue, e.g. by the use of antibodies capable of specifically reacting with the above amino acid sequences.

5       The method, according to this latter aspect, for detection of a nucleic acid sequence which encodes the PSA variant product in a biological sample, comprises the steps of:

(a)   providing a probe comprising at least one of the nucleic acid sequence defined above;

10       (b)   contacting the biological sample with said probe under conditions allowing hybridization of nucleic acid sequences thereby enabling formation of hybridization complexes;

(c)   detecting hybridization complexes, wherein the presence of the complex indicates the presence of nucleic acid sequence encoding the PSA variant  
15   product in the biological sample.

The method as described above is qualitative, i.e. indicates whether the transcript is present in or absent from the sample. The method can also be quantitative, by determining the level of hybridization complexes and then calibrating said levels to determining levels of transcripts of the desired PSA  
20   variant in the sample.

Both qualitative and quantitative determination methods can be used for diagnostic, prognostic and therapy planning purposes.

By a preferred embodiment the probe is part of a nucleic acid chip used for detection purposes, i.e. the probe is a part of an array of probes each present in a  
25   known location on a solid support.

As indicated above the method may be utilized for detecting the presence of prostate cancer, detecting predisposition to prostate cancer, or evaluating the malignancy of prostate cancer, or assessing the development stage of the cancer.

The nucleic acid sequence used in the above method may be a DNA  
30   sequence, an RNA sequence, etc; it may be a coding or a sequence, or a non-coding

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sequence, or a sequence complementary thereto (for respective detection of RNA transcripts or coding-DNA sequences). By quantization of the level of hybridization complexes and calibrating the quantified results it is possible also to detect the level of the transcript in the sample.

5       The probes of the invention may be used to detect polymorphisms (in a specific individual or while screening a population) specifically for pre-disposition to cancer (especially prostate cancer) and loss-of-heterozigosity may be important for monitoring the development of the disease. Detection of disease predisposition or loss of heterozigosity in prostate tissue may be performed on either the coding or  
10 non-coding DNA sequence. One example of such a test is the determination of the exact sequence before position 5620 in GenBank HUMPSANTIG / 1257 of SEQ ID 1 5620 (which is non coding and which contains an additional inserted A as compared to the native PSA), or testing a possible A to G substitution in position 5573 of HUMPSANTIG / 1210 of SEQ ID 1. Both these sites may be indicative of  
15 cancer risk and useful in prognosis.

Methods for detecting mutations in the region coding for the PSA variant product are also provided, which may be methods carried-out in a binary fashion, namely merely detecting whether there is any mismatches between the normal PSA variant nucleic acid sequence and the one present in the sample, or carried-out by  
20 specifically detecting the nature and location of the mutation.

The present invention also concerns a method for detecting PSA variant product in a biological sample, comprising the steps of:

- (a) contacting with said biological sample the antibody of the invention, thereby forming an antibody-antigen complex; and
  - 25       (b) detecting said antibody-antigen complex
- wherein the presence of said antibody-antigen complex correlates with the presence of PSA variant product in said biological sample.

As indicated above, the method can be quantitized to determine the level or the amount of the PSA variant in the sample, alone or in comparison to the level of  
30 the original PSA amino acid sequence from which it was varied, and qualitative

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and quantitative results may be used for diagnostic, prognostic and therapy planning purposes.

By yet another aspect the invention also provides a method for identifying candidate compounds capable of binding to the PSA variant product and  
5 modulating its activity (being either activators or deactivators). The method includes:

- (i) providing a protein or polypeptide comprising an amino acid sequence substantially as depicted in any one of SEQ ID NO: 7 to SEQ ID NO: 12, or a fragment of such a sequence;
- 10 (ii) contacting a candidate compound with said amino acid sequence;
- (iii) measuring the physiological effect of said candidate compound on the activity of the amino acid sequences and selecting those compounds which show a significant effect on said physiological activity.

The activity of the amino acid which should be changed by the modulator  
15 (being either the activator or deactivator) may be for example the binding of the amino acid (PSA variant product) to its native, receptor. Any modulator which changes such an activity has an intersecting potential

The present invention also concerns compounds identified by the above methods described above, which compound may either be an activator of the  
20 serotonin-receptor like product or a deactivator thereof.

## BRIEF DESCRIPTION OF THE DRAWINGS

In order to understand the invention and to see how it may be carried out in practice, a preferred embodiment will now be described, by way of non-limiting  
25 example only, with reference to the accompanying drawings, in which:

Fig. 1 shows the sequence of the original PSA as depicted in HUMPSANTIG. (This is the genomic sequence, which is the same in PSA and PSAL);

Fig. 2 shows the translation of the original PSA sequence;

Fig. 3 shows multiple alignment between the original PSA sequence (termed "*HUMPSANTIG*") and the sequences of the invention (PSAL-O is SEQ ID NO:1; PSAL-1 is SEQ ID NO:2, PSAL-2 is SEQ ID NO:3; PSAL-5 is SEQ ID NO:4 and PSAL-6 is SEQ ID NO:5);

5 Fig. 4 shows a multiple alignment between the 5 PSA splice variant products and the direct translation of the genomic PSA region, depicted here as HUMPSANTIG;

Fig. 5 shows the specific region (signal peptide) of the original PSA that is common with all the splice variants of PSA;

10 Fig. 6 shows a schematic representation of the common locus of the KLK-2 of PSA genes;

Fig. 7 shows a Northern Blot analysis of RNA obtained from various tissues and tested with probes for PSA (left) and probes obtained from SEQ ID NO:2, (termed PSALM in the Figure) (right);

15 Fig. 8 shows a Western Blot analysis of proteins obtained from prostate glands of several patients tested for PSA protein (left) and the PSA variant protein depicted in SEQ ID NO: 2 (right);

Fig. 9 shows immuno-histochemical labeling of human prostate gland with serum of rabbit immunized with PSAL variant peptide of the invention (right) or  
20 unimmunized rabbit;

Fig. 10 shows a cross-section of *in-situ* hybridization of sense and anti-sense probes of PSA (termed "*PSALM*") to tissue obtained from a prostate cancer;

Fig. 11 shows a Western blot analysis of 2 different serum-samples tested for PSA protein PSA (termed "*PSALM*"). The results indicate that the PSA is  
25 secreted to the serum. In the right lane there is a recombinant PSA;

Fig. 12 shows immuno-histochemical labeling of human prostate gland with serum of rabbit immunized with the peptide of the invention (derived from alternative splicing of the KLK-2 gene) (right) or unimmunized rabbit; and

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Fig. 13 shows a Western blot analysis of proteins obtained from prostate glands of 2 different patients (prostate #1 and #2), and from other tissues tested for the protein of the invention derived from the KLK-2 gene.

## 5 DETAILED DESCRIPTION OF A PREFERRED EMBODIMENT

### Example I: PSA variant - nucleic acid sequence

The nucleic acid sequences of the invention include nucleic acid sequences which encode PSA variant product and fragments and analogs thereof. The nucleic acid sequences may alternatively be sequences complementary to the  
10 above coding sequence, or to a region of said coding sequence. The length of the complementary sequence is sufficient to avoid the expression of the coding sequence. The nucleic acid sequences may be in the form of RNA or in the form of DNA, and include messenger RNA, synthetic RNA and DNA, cDNA, and genomic DNA. The DNA may be double-stranded or single-stranded, and if  
15 single-stranded may be the coding strand or the non-coding (anti-sense, complementary) strand. The nucleic acid sequences may also both include dNTPs, rNTPs as well as non naturally occurring sequences. The sequence may also be a part of a hybrid between an amino acid sequence and a nucleic acid sequence.

20 In a general embodiment, the nucleic acid sequence has at least 70%, preferably 80% or 90% sequence identity with the sequence identified as SEQ ID NO:1 to SEQ ID NO:6.

The nucleic acid sequences may include the coding sequence by itself. By another alternative the coding region may be in combination with additional  
25 coding sequences, such as those coding for fusion protein or signal peptides, in combination with non-coding sequences, such as introns and control elements, promoter and terminator elements or 5' and/or 3' untranslated regions, effective for expression of the coding sequence in a suitable host, and/or in a vector or host environment in which the PSA variant nucleic acid sequence is introduced as a  
30 heterologous sequence.

- 20 -

The nucleic acid sequences of the present invention may also have the product coding sequence fused in-frame to a marker sequence which allows for purification of the PSA variant product. The marker sequence may be, for example, a hexahistidine tag to provide for purification of the mature polypeptide fused to the marker in the case of a bacterial host, or, the marker sequence may be a hemagglutinin (HA) tag when a mammalian host, e.g. COS-7 cells, is used. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson, I., *et al. Cell* 37:767 (1984)).

Also included in the scope of the invention are fragments also referred to herein as oligonucleotides, typically having at least 20 bases, preferably 20-30 bases corresponding to a region of the coding-sequence nucleic acid sequence. The fragments may be used as probes, primers, and when complementary also as antisense agents, and the like, according to known methods.

As indicated above, the nucleic acid sequence may be substantially a depicted in SEQ ID NO:1 to SEQ ID NO:6 or fragments thereof or sequences having at least 70%, preferably 70-80%, most preferably 90% identity to the above sequence. Alternatively, due to the degenerative nature of the genetic code, the sequence may be a sequence coding the amino acid sequence of SEQ ID NO:6 to SEQ ID NO:12, or fragments or analogs of said amino acid sequence.

20

#### A. Preparation of nucleic acid sequences

The nucleic acid sequences may be obtained by screening cDNA libraries using oligonucleotide probes which can hybridize to or PCR-amplify nucleic acid sequences which encode the PSA variant products disclosed above. cDNA libraries prepared from a variety of tissues are commercially available and procedures for screening and isolating cDNA clones are well-known to those of skill in the art. Such techniques are described in, for example, Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (2nd Edition), Cold Spring Harbor Press, Plainview, N.Y. and Ausubel FM *et al.* (1989) *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, N.Y.

30

The nucleic acid sequences may be extended to obtain upstream and downstream sequences such as promoters, regulatory elements, and 5' and 3' untranslated regions (UTRs). Extension of the available transcript sequence may be performed by numerous methods known to those of skill in the art, such as PCR or primer extension (Sambrook *et al.*, *supra*), or by the RACE method using, for example, the Marathon RACE kit (Clontech, Cat. # K1802-1).

Alternatively, the technique of "restriction-site" PCR (Gobinda *et al.* *PCR Methods Applic.* 2:318-22, (1993)), which uses universal primers to retrieve flanking sequence adjacent a known locus, may be employed. First, genomic DNA is amplified in the presence of primer to a linker sequence and a primer specific to the known region. The amplified sequences are subjected to a second round of PCR with the same linker primer and another specific primer internal to the first one. Products of each round of PCR are transcribed with an appropriate RNA polymerase and sequenced using reverse transcriptase.

Inverse PCR can be used to amplify or extend sequences using divergent primers based on a known region (Triglia, T. *et al.*, *Nucleic Acids Res.* 16:8186, (1988)). The primers may be designed using OLIGO(R) 4.06 Primer Analysis Software (1992; National Biosciences Inc, Plymouth, Minn.), or another appropriate program, to be 22-30 nucleotides in length, to have a GC content of 50% or more, and to anneal to the target sequence at temperatures about 68-72°C.

The method uses several restriction enzymes to generate a suitable fragment in the known region of a gene. The fragment is then circularized by intramolecular ligation and used as a PCR template.

Capture PCR (Lagerstrom, M. *et al.*, *PCR Methods Applic.* 1:111-19, (1991)) is a method for PCR amplification of DNA fragments adjacent to a known sequence in human and yeast artificial chromosome DNA. Capture PCR also requires multiple restriction enzyme digestions and ligations to place an engineered double-stranded sequence into a flanking part of the DNA molecule before PCR.

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Another method which may be used to retrieve flanking sequences is that of Parker, J.D., *et al.*, *Nucleic Acids Res.*, **19**:3055-60, (1991)). Additionally, one can use PCR, nested primers and PromoterFinder™ libraries to "walk in" genomic DNA (PromoterFinder™; Clontech, Palo Alto, CA). This process avoids the need to screen libraries and is useful in finding intron/exon junctions. Preferred libraries for screening for full length cDNAs are ones that have been size-selected to include larger cDNAs. Also, random primed libraries are preferred in that they will contain more sequences which contain the 5' and upstream regions of genes.

A randomly primed library may be particularly useful if an oligo d(T) library does not yield a full-length cDNA. Genomic libraries are useful for extension into the 5' nontranslated regulatory region.

The nucleic acid sequences and oligonucleotides of the invention can also be prepared by solid-phase methods, according to known synthetic methods. Typically, fragments of up to about 100 bases are individually synthesized, then joined to form continuous sequences up to several hundred bases.

#### **B. Use of PSA variant nucleic acid sequence for the production of PSA variant products**

In accordance with the present invention, nucleic acid sequences specified above may be used as recombinant DNA molecules that direct the expression of PSA variant products.

As will be understood by those of skill in the art, it may be advantageous to produce PSA variant product-encoding nucleotide sequences possessing codons other than those which appear in any one of SEQ ID NO:1 to SEQ ID NO:6 which are those which naturally occur in the human genome. Codons preferred by a particular prokaryotic or eukaryotic host (Murray, E. *et al.* *Nuc Acids Res.*, **17**:477-508, (1989)) can be selected, for example, to increase the rate of PSA variant product expression or to produce recombinant RNA transcripts



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having desirable properties, such as a longer half-life, than transcripts produced from naturally occurring sequence.

The nucleic acid sequences of the present invention can be engineered in order to alter a PSA variant product coding sequence for a variety of reasons, including but not limited to, alterations which modify the cloning, processing and/or expression of the product. For example, alterations may be introduced using techniques which are well known in the art, e.g., site-directed mutagenesis, to insert new restriction sites, to alter glycosylation patterns, to change codon preference, to produce splice variant, etc.

10 The present invention also includes recombinant constructs comprising one or more of the sequences as broadly described above. The constructs comprise a vector, such as a plasmid or viral vector, into which a nucleic acid sequence of the invention has been inserted, in a forward or reverse orientation. In a preferred aspect of this embodiment, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and are commercially available. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are also described in Sambrook, *et al.*, (*supra*).

20 The present invention also relates to host cells which are genetically engineered with vectors of the invention, and the production of the product of the invention by recombinant techniques. Host cells are genetically engineered (i.e., transduced, transformed or transfected) with the vectors of this invention which may be, for example, cloning vectors or expression vectors. The vector may be, 25 for example, in the form of a plasmid, a viral particle, a phage, etc. The engineered host cells can be cultured in conventional nutrient media modified as appropriate for activating promoters, selecting transformants or amplifying the expression of the PSA variant nucleic acid sequence. The culture conditions, such as temperature, pH and the like, are those previously used with the host cell 30 selected for expression, and will be apparent to those skilled in the art.

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The nucleic acid sequences of the present invention may be included in any one of a variety of expression vectors for expressing a product. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences, e.g., derivatives of SV40; bacterial plasmids; phage DNA; baculovirus; yeast  
5 plasmids; vectors derived from combinations of plasmids and phage DNA, viral DNA such as vaccinia, adenovirus, fowl pox virus, and pseudorabies. However, any other vector may be used as long as it is replicable and viable in the host. The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate  
10 restriction endonuclease site(s) by procedures known in the art. Such procedures and related sub-cloning procedures are deemed to be within the scope of those skilled in the art.

The DNA sequence in the expression vector is operatively linked to an appropriate transcription control sequence (promoter) to direct mRNA synthesis.  
15 Examples of such promoters include: LTR or SV40 promoter, the *E.coli lac* or *trp* promoter, the phage lambda *PL* promoter, and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation, and a transcription terminator. The vector may also include  
20 appropriate sequences for amplifying expression. In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in *E.coli*.

25 The vector containing the appropriate DNA sequence as described above, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein. Examples of appropriate expression hosts include: bacterial cells, such as *E.coli*, *Streptomyces*, *Salmonella typhimurium*; fungal cells, such as yeast; insect cells  
30 such as *Drosophila* and *Spodoptera Sf9*; animal cells such as CHO, COS, HEK

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293 or Bowes melanoma; adenoviruses; plant cells, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein. The invention is not limited by the host cells employed.

In bacterial systems, a number of expression vectors may be selected depending upon the use intended for the PSA variant product. For example, when large quantities of PSA variant product are needed for the induction of antibodies, vectors which direct high level expression of fusion proteins that are readily purified may be desirable. Such vectors include, but are not limited to, multifunctional *E.coli* cloning and expression vectors such as *Bluescript*(R) (Stratagene), in which the PSA variant polypeptide coding sequence may be ligated into the vector in-frame with sequences for the amino-terminal Met and the subsequent 7 residues of beta-galactosidase so that a hybrid protein is produced; *pIN* vectors (Van Heeke & Schuster *J. Biol. Chem.* **264**:5503-5509, (1989)); *pET* vectors (Novagen, Madison WI); and the like.

In the yeast *Saccharomyces cerevisiae* a number of vectors containing constitutive or inducible promoters such as alpha factor, alcohol oxidase and PGH may be used. For reviews, see Ausubel *et al.* (*supra*) and Grant *et al.*, (*Methods in Enzymology* **153**:516-544, (1987)).

In cases where plant expression vectors are used, the expression of a sequence encoding PSA variant product may be driven by any of a number of promoters. For example, viral promoters such as the 35S and 19S promoters of *CaMV* (Brisson *et al.*, *Nature* **310**:511-514. (1984)) may be used alone or in combination with the omega leader sequence from TMV (Takamatsu *et al.*, *EMBO J.*, **6**:307-311, (1987)). Alternatively, plant promoters such as the small subunit of RUBISCO (Coruzzi *et al.*, *EMBO J.* **3**:1671-1680, (1984); Broglie *et al.*, *Science* **224**:838-843, (1984)); or heat shock promoters (Winter J and Sinibaldi R.M., *Results Probl. Cell Differ.*, **17**:85-105, (1991)) may be used. These constructs can be introduced into plant cells by direct DNA transformation or pathogen-mediated transfection. For reviews of such techniques, see Hobbs S. or Murry L.E. (1992) in McGraw Hill Yearbook of Science and Technology,

McGraw Hill, New York, N.Y., pp 191-196; or Weissbach and Weissbach (1988) *Methods for Plant Molecular Biology*, Academic Press, New York, N.Y., pp 421-463.

PSA variant product may also be expressed in an insect system. In one  
5 such system, Autographa californica nuclear polyhedrosis virus (AcNPV) is used  
as a vector to express foreign genes in *Spodoptera frugiperda* cells or in  
*Trichoplusia* larvae. The PSA variant product coding sequence may be cloned  
into a nonessential region of the virus, such as the polyhedrin gene, and placed  
under control of the polyhedrin promoter. Successful insertion of PSA variant  
10 coding sequence will render the polyhedrin gene inactive and produce  
recombinant virus lacking coat protein coat. The recombinant viruses are then  
used to infect *S. frugiperda* cells or *Trichoplusia* larvae in which PSA variant  
protein is expressed (Smith *et al.*, *J. Virol.* 46:584, (1983); Engelhard, E.K. *et al.*,  
*Proc. Nat. Acad. Sci.* 91:3224-7, (1994)).

15 In mammalian host cells, a number of viral-based expression systems may  
be utilized. In cases where an adenovirus is used as an expression vector, a PSA  
variant product coding sequence may be ligated into an adenovirus  
transcription/translation complex consisting of the late promoter and tripartite  
leader sequence. Insertion in a nonessential E1 or E3 region of the viral genome  
20 will result in a viable virus capable of expressing PSA variant protein in infected  
host cells (Logan and Shenk, *Proc. Natl. Acad. Sci.* 81:3655-59, (1984). In  
addition, transcription enhancers, such as the Rous sarcoma virus (RSV)  
enhancer, may be used to increase expression in mammalian host cells.

Specific initiation signals may also be required for efficient translation of  
25 a PSA variant protein coding sequence. These signals include the ATG initiation  
codon and adjacent sequences. In cases where PSA variant product coding  
sequence, its initiation codon and upstream sequences are inserted into the  
appropriate expression vector, no additional translational control signals may be  
needed. However, in cases where only coding sequence, or a portion thereof, is  
30 inserted, exogenous transcriptional control signals including the ATG initiation

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codon must be provided. Furthermore, the initiation codon must be in the correct reading frame to ensure transcription of the entire insert. Exogenous transcriptional elements and initiation codons can be of various origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of enhancers appropriate to the cell system in use (Scharf, D. *et al.*, (1994) *Results Probl. Cell Differ.*, 20:125-62, (1994); Bittner et al., *Methods in Enzymol* 153:516-544, (1987)).

In a further embodiment, the present invention relates to host cells containing the above-described constructs. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-Dextran mediated transfection, or electroporation (Davis, L., Dibner, M., and Battey, I. (1986) *Basic Methods in Molecular Biology*). Cell-free translation systems can also be employed to produce polypeptides using RNAs derived from the DNA constructs of the present invention.

A host cell strain may be chosen for its ability to modulate the expression of the inserted sequences or to process the expressed protein in the desired fashion. Such modifications of the protein include, but are not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation and acylation. Post-translational processing which cleaves a "pre-pro" form of the protein may also be important for correct insertion, folding and/or function. Different host cells such as CHO, HeLa, MDCK, 293, WI38, etc. have specific cellular machinery and characteristic mechanisms for such post-translational activities and may be chosen to ensure the correct modification and processing of the introduced, foreign protein.

For long-term, high-yield production of recombinant proteins, stable expression is preferred. For example, cell lines which stably express PSA variant product may be transformed using expression vectors which contain viral origins

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of replication or endogenous expression elements and a selectable marker gene. Following the introduction of the vector, cells may be allowed to grow for 1-2 days in an enriched media before they are switched to selective media. The purpose of the selectable marker is to confer resistance to selection, and its  
5 presence allows growth and recovery of cells which successfully express the introduced sequences. Resistant clumps of stably transformed cells can be proliferated using tissue culture techniques appropriate to the cell type.

Any number of selection systems may be used to recover transformed cell lines. These include, but are not limited to, the herpes simplex virus thymidine  
10 kinase (Wigler M., *et al.*, *Cell* 11:223-32, (1977)) and adenine phosphoribosyltransferase (Lowy I., *et al.*, *Cell* 22:817-23, (1980)) genes which can be employed in *tk-* or *aprt-* cells, respectively. Also, antimetabolite, antibiotic or herbicide resistance can be used as the basis for selection; for example, *dhfr* which confers resistance to methotrexate (Wigler M., *et al.*, *Proc.*  
15 *Natl. Acad. Sci.* 77:3567-70, (1980)); *npt*, which confers resistance to the aminoglycosides neomycin and G-418 (Colbere-Garapin, F. *et al.*, *J. Mol. Biol.*, 150:1-14, (1981)) and *als* or *pat*, which confer resistance to chlorsulfuron and phosphinotricin acetyltransferase, respectively (Murry, *supra*). Additional selectable genes have been described, for example, *trpB*, which allows cells to  
20 utilize indole in place of tryptophan, or *hisD*, which allows cells to utilize histinol in place of histidine (Hartman S.C. and R.C. Mulligan, *Proc. Natl. Acad. Sci.* 85:8047-51, (1988)). The use of visible markers has gained popularity with such markers as anthocyanins, beta-glucuronidase and its substrate, GUS, and luciferase and its substrates, luciferin and ATP, being widely used not only to  
25 identify transformants, but also to quantify the amount of transient or stable protein expression attributable to a specific vector system (Rhodes, C.A. *et al.*, *Methods Mol. Biol.*, 55:121-131, (1995)).

Host cells transformed with a nucleotide sequence encoding PSA variant product may be cultured under conditions suitable for the expression and  
30 recovery of the encoded protein from cell culture. The product produced by a

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recombinant cell may be secreted or contained intracellularly depending on the sequence and/or the vector used. As will be understood by those of skill in the art, expression vectors containing nucleic acid sequences encoding PSA variant product can be designed with signal sequences which direct secretion of PSA  
5 variant product through a prokaryotic or eukaryotic cell membrane.

PSA variant product may also be expressed as a recombinant protein with one or more additional polypeptide domains added to facilitate protein purification. Such purification facilitating domains include, but are not limited to, metal chelating peptides such as histidine-tryptophan modules that allow  
10 purification on immobilized metals, protein A domains that allow purification on immobilized immunoglobulin, and the domain utilized in the FLAGS extension/affinity purification system (Immunex Corp, Seattle, Wash.). The inclusion of a protease-cleavable polypeptide linker sequence between the purification domain and PSA variant protein is useful to facilitate purification.

15 One such expression vector provides for expression of a fusion protein comprising a PSA variant polypeptide fused to a polyhistidine region separated by an enterokinase cleavage site. The histidine residues facilitate purification on IMIAC (immobilized metal ion affinity chromatography, as described in Porath, *et al.*, *Protein Expression and Purification*, 3:263-281,  
20 (1992)) while the enterokinase cleavage site provides a means for isolating PSA variant polypeptide from the fusion protein. *pGEX* vectors (Promega, Madison, Wis.) may also be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption to ligand-agarose beads  
25 (e.g., glutathione-agarose in the case of GST-fusions) followed by elution in the presence of free ligand.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced by appropriate means (e.g., temperature shift or chemical induction) and cells are  
30 cultured for an additional period. Cells are typically harvested by centrifugation,

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disrupted by physical or chemical means, and the resulting crude extract retained for further purification. Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents, or other methods, which are well known to those skilled in the art.

The PSA variant products can be recovered and purified from recombinant cell cultures by any of a number of methods well known in the art, including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography, and lectin chromatography. Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps.

15

#### **C. Diagnostic applications utilizing nucleic acid sequences**

The nucleic acid sequences of the present invention may be used for a variety of diagnostic purposes. The nucleic acid sequences may be used to detect and quantitate expression of PSA variant in patient's cells, e.g. biopsied tissues, by detecting the presence of mRNA coding for PSA variant product. Alternatively, the assay may be used to detect free PSA variant in the serum or blood. This assay typically involves obtaining total mRNA from the tissue or serum and contacting the mRNA with a nucleic acid probe. The probe is a nucleic acid molecule of at least 20 nucleotides, preferably 20-30 nucleotides, capable of specifically hybridizing with a sequence included within the sequence of a nucleic acid molecule encoding PSA variant under hybridizing conditions, detecting the presence of mRNA hybridized to the probe, and thereby detecting the expression of PSA variant. This assay can be used to distinguish between absence, presence, and excess expression of PSA variant product and to monitor levels of PSA variant expression during therapeutic intervention.

30



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The invention also contemplates the use of the nucleic acid sequences as a diagnostic for diseases resulting from inherited defective PSA variant sequences.

These sequences can be detected by comparing the sequences of the defective (i.e., mutant) PSA variant coding region with that of a normal coding region.

5 Association of the sequence coding for mutant PSA variant product with abnormal PSA variant product activity may be verified. In addition, sequences encoding mutant PSA variant products can be inserted into a suitable vector for expression in a functional assay system (e.g., colorimetric assay, complementation experiments in a PSA variant protein deficient strain of  
10 HEK293 cells) as yet another means to verify or identify mutations. Once mutant genes have been identified, one can then screen populations of interest for carriers of the mutant gene.

Individuals carrying mutations in the nucleic acid sequence of the present invention may be detected at the DNA level by a variety of techniques. Nucleic  
15 acids used for diagnosis may be obtained from a patient's cells, including but not limited to such as from blood, urine, saliva, placenta, tissue biopsy and autopsy material and in particular tissue obtained from the prostate gland. Genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR (Saiki, *et al.*, *Nature* 324:163-166, (1986)) prior to analysis. RNA or cDNA  
20 may also be used for the same purpose. As an example, PCR primers complementary to the nucleic acid of the present invention can be used to identify and analyze mutations in the gene of the present invention. Deletions and insertions can be detected by a change in size of the amplified product in comparison to the normal genotype.

25 Point mutations can be identified by hybridizing amplified DNA to radiolabeled RNA of the invention or alternatively, radiolabeled antisense DNA sequences of the invention. Sequence changes at specific locations may also be revealed by nuclease protection assays, such as RNase and S1 protection or the chemical cleavage method (e.g. Cotton, *et al.* *Proc. Natl. Acad. Sci. USA*,  
30 85:4397-4401, (1985)), or by differences in melting temperatures. "Molecular

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*beacons*" (Kostrikis L.G. *et al.*, Science 279:1228-1229, (1998)), hairpin-shaped, single-stranded synthetic oligo- nucleotides containing probe sequences which are complementary to the nucleic acid of the present invention, may also be used to detect point mutations or other sequence changes as well as monitor expression levels of PSA variant product.

Another method for detecting mutations uses two DNA probes which are designed to hybridize to adjacent regions of a target, with abutting bases, where the region of known or suspected mutation(s) is at or near the abutting bases. The two probes may be joined at the abutting bases, e.g., in the presence of a ligase enzyme, but only if both probes are correctly base paired in the region of probe junction. The presence or absence of mutations is then detectable by the presence or absence of ligated probe.

Also suitable for detecting mutations in the PSA variant product coding sequence are oligonucleotide array methods based on sequencing by hybridization (SBH), as described, for example, in U.S. Patent No. 5,547,839. In a typical method, the DNA target analyte is hybridized with an array of oligonucleotides formed on a microchip. The sequence of the target can then be "read" from the pattern of target binding to the array.

#### 20 D. *In situ* hybridization using probes of PSA

*In-situ* hybridisation was carried out according to the procedure described in the Boehringer-Mannheim's publication "Non-Radioative *In-Situ* Hybridization Application Manual", 2<sup>nd</sup> edition, 1996. Labelling was carried out according to Chapter 4, section V, and hybridization according to Chapter 5, section IV. Slides were prepared in paraffin and treated according to the procedures described in Chapter 2. The probe used was derived from the PSAL-1 sequence (SEQ ID NO: 2). The anti-sense probe was used to detect the presence of PSA variant mRNA, and the sense probe was used as control. Results in Figure 10 indicates high-level expression of the PSA variant mRNA in prostate epithelial lumen cells.

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### E. Therapeutic applications of nucleic acid sequences

Nucleic acid sequences of the invention may also be used for therapeutic purposes. Turning first to the anti-PSA variant aspect, expression of PSA variant product may be modulated through antisense technology, which controls gene  
5 expression through hybridization of complementary nucleic acid sequences, i.e. antisense DNA or RNA, to the control, 5' or regulatory regions of the gene encoding PSA variant product. For example, the 5' coding portion of the nucleic acid sequence sequence which codes for the product of the present invention is used to design an antisense oligonucleotide of from about 10 to 40 base pairs in  
10 length. Oligonucleotides derived from the transcription start site, e.g. between positions -10 and +10 from the start site, are preferred. An antisense DNA oligonucleotide is designed to be complementary to a region of the nucleic acid sequence involved in transcription (Lee *et al.*, *Nucl. Acids, Res.*, 6:3073, (1979); Cooney *et al.*, *Science* 241:456, (1988); and Dervan *et al.*, *Science* 251:1360,  
15 (1991)), thereby preventing transcription and the production of the PSA variant products. An antisense RNA oligonucleotide hybridizes to the mRNA *in vivo* and blocks translation of the mRNA molecule into the PSA variant products (Okano *J. Neurochem.* 56:560, (1991)). The antisense constructs can be delivered to cells by procedures known in the art such that the antisense RNA or DNA may be  
20 expressed *in vivo*. The antisense may be antisense mRNA or DNA sequence capable of coding such antisense mRNA. The antisense mRNA or the DNA coding thereof can be complementary to the full sequence of nucleic acid sequences coding to the PSA variant protein or to a fragment of such a sequence which is sufficient to inhibit production of a protein product.

25 Turning now to the PSA variant aspect, expression of PSA variant product may be increased by providing coding sequences for coding for said product under the control of suitable control elements ending its expression in the desired host.

The nucleic acid sequences of the invention may be employed in  
30 combination with a suitable pharmaceutical carrier. Such compositions comprise

a therapeutically effective amount of the compound, and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The formulation should suit the mode of administration.

5       The polypeptides, and activator and deactivator compounds (see below), which are polypeptides, may also be employed in accordance with the present invention by expression of such polypeptides *in vivo*, which is often referred to as "*gene therapy*." Cells from a patient may be engineered with a nucleic acid sequence (DNA or RNA) encoding a polypeptide *ex vivo*, with the engineered  
10 cells then being provided to a patient to be treated with the polypeptide. Such methods are well-known in the art. For example, cells may be engineered by procedures known in the art by use of a retroviral particle containing RNA encoding a polypeptide of the present invention.

Similarly, cells may be engineered *in vivo* for expression of a polypeptide  
15 *in vivo* by procedures known in the art. As known in the art, a producer cell for producing a retroviral particle containing RNA encoding the polypeptide of the present invention may be administered to a patient for engineering cells *in vivo* and expression of the polypeptide *in vivo*. These and other methods for administering a product of the present invention by such method should be  
20 apparent to those skilled in the art from the teachings of the present invention. For example, the expression vehicle for engineering cells may be other than a retrovirus, for example, an adenovirus which may be used to engineer cells *in vivo* after combination with a suitable delivery vehicle.

Retroviruses from which the retroviral plasmid vectors mentioned above  
25 may be derived include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, gibbon ape leukemia virus, human immunodeficiency virus, adenovirus, Myeloproliferative Sarcoma Virus, and mammary tumor virus.

The retroviral plasmid vector is employed to transduce packaging cell  
30 lines to form producer cell lines. Examples of packaging cells which may be

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transfected include, but are not limited to, the *PE501*, *PA317*, *psi-2*, *psi-AM*, *PA12*, *T19-14X*, *VT-19-17-H2*, *psi-CRE*, *psi-CRIP*, *GP+E-86*, *GP+envAml2*, and *DAN* cell lines as described in Miller (*Human Gene Therapy*, Vol. 1, pg. 5-14, (1990)). The vector may transduce the packaging cells through any means  
5 known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and  $\text{CaPO}_4$  precipitation. In one alternative, the retroviral plasmid vector may be encapsulated into a liposome, or coupled to a lipid, and then administered to a host.

The producer cell line generates infectious retroviral vector particles  
10 which include the nucleic acid sequence(s) encoding the polypeptides. Such retroviral vector particles then may be employed, to transduce eukaryotic cells, either in vitro or in vivo. The transduced eukaryotic cells will express the nucleic acid sequence(s) encoding the polypeptide. Eukaryotic cells which may be transduced include, but are not limited to, embryonic stem cells, embryonic  
15 carcinoma cells, as well as hematopoietic stem cells, hepatocytes, fibroblasts, myoblasts, keratinocytes, endothelial cells, and bronchial epithelial cells.

The genes introduced into cells may be placed under the control of inducible promoters, such as the radiation-inducible Egr-1 promoter, (Maceri, H.J., *et al.*, *Cancer Res.*, 56(19):4311 (1996)), to stimulate PSA variant  
20 production or antisense inhibition in response to radiation, eg., radiation therapy for treating tumors.

#### **F. Northern Blot Analysis**

RNA samples were obtained from spleen, thymus, prostate, testis, ovary, small  
25 intestine, colon and leukocytes electrophoresed through a 1.5% agarose gel containing formaldehyde and transferred onto nylon (Hybond-N, Amersham) paper (Thomas, 1980). Prehybridization was for 2 hours in a buffer containing 10% Dextrane Sulfate, 1M NaCl and 1% SDS, at 65°C. Hybridization was in the same buffer with  $5 \times 10^6$  cpm of the appropriate probe at 65°C for 18 hours. After  
30 one wash in 2XSSC, 0.1% SDS for 15 minutes at 65°C and several washes in

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0.2XSSC, 0.1% SDS at 65°C the filter was exposed to an X-ray film. Phosphorimager analysis was performed as well. The results are shown in Fig. 7.

As can be seen with the PSA probe (left), a single band was detected, while with the probe of the invention (termed "*PSAL*") (derived from SEQ ID 2, in the common region with SEQ IDs 3, 4, and 5) several bands were detected in prostate tissue, which indicates the presence of different PSA splice variants.

### Example II. PSA variant product

The substantially purified PSA variant product of the invention has been defined above as the product coded from the nucleic acid sequence of the invention. Preferably the amino acid sequence is an amino acid sequence having at least 90% identity to the sequence identified as any one of SEQ ID NO: 7 to SEQ. ID NO. 12. The protein may be in mature and/or modified form, also as defined above. Also contemplated are protein fragments having at least 10 contiguous amino acid residues, preferably at least 10-20 residues, derived from the PSA variant protein.

The sequence variations are preferably those that are considered conserved substitutions, as defined above. Thus, for example, a protein with a sequence having at least 80% sequence identity with the protein identified as SEQ ID NO: 7 to SEQ ID NO: 12, preferably by utilizing conserved substitutions as defined above. In a more specific embodiment, the protein has or contains the sequence identified SEQ ID NO: 7 to SEQ. ID NO: 12. The PSA variant product may be (i) one in which one or more of the amino acid residues in a sequence listed above are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue), or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the PSA variant product is fused with another compound, such as a compound to increase the half-life of the protein (for example, polyethylene glycol (PEG)), or a moiety which serves as targeting means to direct the protein to its target tissue or target cell population (such as an antibody), or (iv) one in which additional amino acids

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are fused to the PSA variant product. Such fragments, variant and derivatives are deemed to be within the scope of those skilled in the art from the teachings herein.

5    **A.    Preparation of PSA variant product**

Recombinant methods for producing and isolating the PSA variant product, and fragments of the protein are described above.

In addition to recombinant production, fragments and portions of PSA variant product may be produced by direct peptide synthesis using solid-phase  
10 techniques (cf. Stewart *et al.*, (1969) Solid-Phase Peptide Synthesis, WH Freeman Co, San Francisco; Merrifield J., *J. Am. Chem. Soc.*, 85:2149-2154, (1963)). In vitro peptide synthesis may be performed using manual techniques or by automation. Automated synthesis may be achieved, for example, using Applied Biosystems 431A Peptide Synthesizer (Perkin Elmer, Foster City, Calif.)  
15 in accordance with the instructions provided by the manufacturer. Fragments of PSA variant product may be chemically synthesized separately and combined using chemical methods to produce the full length molecule.

**B.    Western Blot Analysis**

20    Western Blot analysis was performed according to procedures well known in the art, that are described in the Maniatis Laboratory Manual. Post processing was performed using PIERCE SuperSignal staining kit.

CG-47, CG-30, CG-23, and CG-35 are all hyperplastic prostate tissue sample. CG-37 is a normal prostate sample. The results indicate that the original  
25 PSA is expressed in hyperplastic, but not normal, prostate tissue (right) while the PSA variant (denoted PSALM) is weakly present hyperplastic prostate and highly expressed in normal tissue. Neither molecule is present in detectable levels in the spleen control.

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**C. Therapeutic uses and compositions utilizing the PSA variant product**

The PSA variant product of the invention is generally useful in treating diseases and disorders which are characterized by a lower than normal level of PSA variant expression, and or diseases which can be cured or ameliorated by raising the level of the PSA variant product, even if the level is normal.

PSA variant products or fragments may be administered by any of a number of routes and methods designed to provide a consistent and predictable concentration of compound at the target organ or tissue. The product-containing compositions may be administered alone or in combination with other agents, such as stabilizing compounds, and/or in combination with other pharmaceutical agents such as drugs or hormones.

PSA variant product-containing compositions may be administered by a number of routes including, but not limited to oral, intravenous, intramuscular, transdermal, subcutaneous, topical, sublingual, or rectal means as well as by nasal application. PSA variant product-containing compositions may also be administered via liposomes. Such administration routes and appropriate formulations are generally known to those of skill in the art.

The product can be given via intravenous or intraperitoneal injection. Similarly, the product may be injected to other localized regions of the body. The product may also be administered via nasal insufflation. Enteral administration is also possible. For such administration, the product should be formulated into an appropriate capsule or elixir for oral administration, or into a suppository for rectal administration.

The foregoing exemplary administration modes will likely require that the product be formulated into an appropriate carrier, including ointments, gels, suppositories. Appropriate formulations are well known to persons skilled in the art.

Dosage of the product will vary, depending upon the potency and therapeutic index of the particular polypeptide selected.



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A therapeutic composition for use in the treatment method can include the product in a sterile injectable solution, the polypeptide in an oral delivery vehicle, the product in an aerosol suitable for nasal administration, or the product in a nebulized form, all prepared according to well known methods. Such compositions comprise a therapeutically effective amount of the compound, and a pharmaceutically acceptable carrier or excipient. Such a carrier includes but is not limited to saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof.

### 10 **Example III. Screening methods for activators and deactivators**

The present invention also includes an assay for identifying molecules, such as synthetic drugs, antibodies, peptides, or other molecules, which have a modulating effect on the activity of the PSA variant product, e.g. activators or deactivators of the PSA variant product of the present invention. Such an assay comprises the steps of providing an PSA variant product encoded by the nucleic acid sequences of the present invention, contacting the PSA variant protein with one or more candidate molecules to determine the candidate molecules modulating effect on the activity of the PSA variant product, and selecting from the molecules a candidate's molecule capable of modulating PSA variant product physiological activity.

PSA variant product, its catalytic or immunogenic fragments or oligopeptides thereof, can be used for screening therapeutic compounds in any of a variety of drug screening techniques. The fragment employed in such a test may be free in solution, affixed to a solid support, borne on a cell membrane or located intracellularly. The formation of binding complexes, between PSA variant product and the agent being tested, may be measured. Alternatively, the activator or deactivator may work by serving as agonist or antagonist, respectively, of the PSA variant receptor and their effect may be determined in connection with the receptor.

- 40 -

Another technique for drug screening which may be used provides for high throughput screening of compounds having suitable binding affinity to the PSA variant product is described in detail by Geysen in PCT Application WO 84/03564, published on Sep. 13, 1984. In summary, large numbers of different  
5 small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The peptide test compounds are reacted with the full PSA variant product or with fragments of PSA variant product and washed. Bound PSA variant product is then detected by methods well known in the art. Substantially purified PSA variant product can also be coated directly onto plates  
10 for use in the aforementioned drug screening techniques. Alternatively, non-neutralizing antibodies can be used to capture the peptide and immobilize it on a solid support.

Antibodies to the PSA variant product, as described in Example IV below, may also be used in screening assays according to methods well known in the art.  
15 For example, a "*sandwich*" assay may be performed, in which an anti-PSA variant antibody is affixed to a solid surface such as a microtiter plate and PSA variant product is added. Such an assay can be used to capture compounds which bind to the PSA variant product. Alternatively, such an assay may be used to measure the ability of compounds to influence with the binding of PSA variant  
20 product to the PSA variant receptor, and then select those compounds which effect the binding.

#### **Example IV. Anti-PSA variant antibodies**

##### **A. Synthesis**

25 In still another aspect of the invention, the purified PSA variant product is used to produce anti-PSA variant antibodies which have diagnostic and therapeutic uses related to the activity, distribution, and expression of the PSA variant product, in particular diagnostic application in identification of prostate cancer, (distinguishing between malignant and benign states) and as targeting  
30 means for delivery of cytotoxic compounds to tumor cells.

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Antibodies to PSA variant product may be generated by methods well known in the art. Such antibodies may include, but are not limited to, polyclonal, monoclonal, chimeric, humanized, single chain, Fab fragments and fragments produced by an Fab expression library. Antibodies, i.e., those which inhibit dimer formation, are especially preferred for therapeutic use.

PSA variant product for antibody induction does not require biological activity; however, the protein fragment or oligopeptide must be antigenic. Peptides used to induce specific antibodies may have an amino acid sequence consisting of at least five amino acids, preferably at least 10 amino acids of the sequences specified in any of the 7 to 12 SEQ ID NO. Preferably they should mimic a portion of the amino acid sequence of the natural protein and may contain the entire amino acid sequence of a small, naturally occurring molecule. The antibodies may also distinguish antibodies, i.e. antibodies which bind to an amino acid sequence present in the PSA variant and not in the original PSA sequence. For the production of said distinguishing antibodies "*distinguishing amino acid sequences*" may be used for example having the sequence CQAELSPPTQHSPDREL.

Antibodies may also be produced by inducing *in vivo* production in the lymphocyte population or by screening recombinant immunoglobulin libraries or panels of highly specific binding reagents as disclosed in Orlandi *et al.* (*Proc. Natl. Acad. Sci.* **86**:3833-3837, 1989)), and Winter G and Milstein C., (*Nature* **349**:293-299, (1991)).

Antibody fragments which contain specific binding sites for PSA variant protein may also be generated. For example, such fragments include, but are not limited to, the F(ab')<sub>2</sub> fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the F(ab')<sub>2</sub> fragments. Alternatively, Fab expression libraries may be constructed to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity (Huse W.D. *et al.*, *Science* **256**:1275-1281, (1989)).

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### Production of Antibodies

Human and mouse cDNA fragments were subcloned into the pET-28(a-c) vectors (Novagen, USA). DNA was prepared from positive clones and introduced into the E. coli strain DE3 according to the manufacturer's recommendations. After  
5 induction, extracts were electrophoresed through a 10% SDS-PAGE. Extracts were prepared from clones that expressed the expected size protein and loaded on a nickel-agarose column. The His containing proteins were isolated from the column according to the manufacturer's recommendations and used in injections. Polyclonal antibodies against human PSAL peptide were prepared by immunizing  
10 rabbits with 3-4 injections of 0.5 mg of the purified protein at 1-2 week intervals. Animals were bled 10 days after the final booster. Serum was separated from the blood and stored at -80C. The peptide defined above as "distinguishing amino acid sequence" was used for immunization.

### 15 B. Diagnostic applications of antibodies

A variety of protocols for competitive binding or immunoradiometric assays using either polyclonal or monoclonal antibodies with established specificities are well known in the art. Such immunoassays typically involve the formation of complexes between PSA variant product and its specific antibody  
20 and the measurement of complex formation. A two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on a specific PSA variant product is preferred, but a competitive binding assay may also be employed. These assays are described in Maddox D.E., et al., (J. Exp. Med. 158:1211, (1983)).

25 Antibodies which specifically bind PSA variant product are useful for the diagnosis of conditions or diseases characterized by expression of PSA variant protein, in particular prostate cancer. Alternatively, such antibodies may be used in assays to monitor patients being treated with PSA variant product, its activators, or its deactivators. Diagnostic assays for PSA variant protein include  
30 methods utilizing the antibody and a label to detect PSA variant product in

human body fluids or extracts of cells or tissues. The products and antibodies of the present invention may be used with or without modification. Frequently, the proteins and antibodies will be labeled by joining them, either covalently or noncovalently, with a reporter molecule. A wide variety of reporter molecules  
5 are known in the art.

A variety of protocols for measuring PSA variant product, using either polyclonal or monoclonal antibodies specific for the respective protein are known in the art. Examples include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), and fluorescent activated cell sorting (FACS). As  
10 noted above, a two-site, monoclonal-based immunoassay utilizing monoclonal antibodies reactive to two non-interfering epitopes on PSA variant product is preferred, but a competitive binding assay may be employed. These assays are described, among other places, in Maddox, *et al. (supra)*. Such protocols provide a basis for diagnosing altered or abnormal levels of PSA variant product  
15 expression. Normal or standard values for PSA variant product expression are established by combining body fluids or cell extracts taken from normal subjects, preferably human, with antibody to PSA variant product under conditions suitable for complex formation which are well known in the art. The amount of standard complex formation may be quantified by various methods, preferably by  
20 photometric methods. Then, standard values obtained from normal samples may be compared with values obtained from samples from subjects potentially affected by disease. Deviation between standard and subject values establishes the presence of disease state.

The antibody assays are useful to determine the level of PSA variant  
25 present in a body fluid sample, or in a particular tissue, e.g., biopsied tumor tissue, for example from the prostate gland, as an indication of whether PSA variant is being overexpressed or underexpressed in the tissue, or as an indication of how PSA variant levels are responding to drug treatment.

### C. Immunohistochemical staining:

Human prostate micron sections were prepared using a R.Gung microtome and fixed on slides pretreated with 2% Tespa (Sigma, USA). Deparaffinization was performed for 30 minutes at 80°C. Hydration was executed by immersing the slides twice in xylene (5 minutes each), twice in 100% ethanol (5 minutes each), twice in 95% ethanol (5 minutes each), once in 70% ethanol (5 minutes), and once in PBS pH 7.4 (10 minutes). After incubation in 50  $\mu$ l/slide of 1.5 mg/ml hyaluronidase in PBS pH 6.5 for 1 hour at 37°C the slides were washed in PBS pH 7.4 (10 minutes). 50  $\mu$ l/slide of 0.3% H<sub>2</sub>O<sub>2</sub> in PBS pH 7.4 were added for 10 minutes after which they were washed in PBS pH 7.4 (10 minutes).

Blocking was performed by addition of 50  $\mu$ l/slide of normal goat serum containing 20% trasylol at 37°C for 10 minutes. Rabbit anti-testis antibodies were diluted 1:50 in 10% blocking solution (normal goat serum containing 20% trasylol (Bayer, Germany) and interacted with the slides for 18 hours at 4°C. Then the slides were washed 3 times in PBS pH 7.4 and immersed for 10 minutes in PBS pH 7.4. Horse raddish peroxidase (HRP) conjugated goat anti rabbit antibodies (Sigma, USA) in PBS pH 7.4 containing 20% trasylol (Bayer, Germany) diluted 1:40, were added for 30 minutes at room temperature, in the dark, following by wash in PBS pH 7.4 for 10 minutes. For HRP reaction 0.4 mg/ml of the substrate (3'3' diaminobenzoidin) was added in the dark for 10 minutes. Following 3 washes in PBS pH 7.4 and immersion for 10 minutes in PBS pH 7.4, staining of the slides was performed with 1% methylene blue in PBS pH 7.4 for 5 min. Following two washes in water, dehydration was carried out by immersing the slides 3 times in 70% ethanol, 3 times in 95% ethanol, 6 times in 100% ethanol and 6 times in xylene. Mounting was performed with Mercoglass (Merk, USA). The results are shown in Fig. 9. The result indicate a high presence level of the PSA variant protein in the lumen-lining of prostate epithelial cells (color gold, left picture, vs. the pre-immune serum control on the right picture, where no gold color is detectable.)

#### D. Therapeutic uses of antibodies

In addition to their diagnostic use the antibodies may have a therapeutical utility in blocking or decreasing the activity of the PSA variant product in pathological conditions where its activity or concentration are too high, for example in prostate cancer. In addition, the antibodies may be conjugated to cytotoxic compounds and thus may serve as means for targeting the cytotoxic moiety only to cancer cells which express membrane-bound PSA variant product.

The antibody employed is preferably a humanized monoclonal antibody, or a human Mab produced by known globulin-gene library methods. The antibody is administered typically as a sterile solution by IV injection, although other parenteral routes may be suitable. Typically, the antibody is administered in an amount between about 1-15 mg/kg body weight of the subject. Treatment is continued, e.g., with dosing every 1-7 days, until a therapeutic improvement is seen.

Although the invention has been described with reference to specific methods and embodiments, it is appreciated that various modifications and changes may be made without departing from the invention.

#### EXAMPLE V. Immuno-histochemistry

Immunohistochemical staining was performed using Histostain sp kit (Zymed Laboratories INC.).

Human prostate micron sections were prepared using a R. Gung microtome and fixed on superfrost slides with 2% Tespa. Deparaffinization was performed for 10 mins. at xylen. Hydration three times 100% ethanol and once 95% ethanol. The slides were washed in Ddw, following incubation with 3% H<sub>2</sub>O<sub>2</sub> for 5 mins. After incubation the slides were washed twice in ddw, and twice in 0.05M Tris Hcl Ph 7.6 (optimax wash buffer, BioGenex).

Blocking was performed with serum blocking solution (ready to use, reagent A, Zymed) 100 ul each slide incubate 10 mins. Primary antibody was

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diluted 1:50 in antibody diluent reagent solution (Zymed), and incubated in moist chamber with the slides for 1 hour.

Following washing (three times in optimax buffer) the slides were incubated with 100  $\mu$ l biotinylated second antibody ready to use (reagent B  
5 Zymed), for 10 mins, then washed three more times in optimax buffer. The slides were incubated with 100  $\mu$ l enzyme conjugate HRP-streptavidin ready to use (reagent C, Zymed) for 10 mins, and washed twice in optimax buffer. Then 100  $\mu$ l substrate (liquid DAB substrate, Zymed) were added for 3 mins.

Following the incubation with the substrate, the slides were washed twice  
10 in ddw and stained with Hematoxylen solution (Zymed) for 2 mins. Then the slides were washed in tap water for 1 hour. The dehydration was carried out by immersing the slides 2 times in 95% ethanol, 3 times in 100% ethanol, 3 times in Zylen. Mounting was performed with mounting solution (Zymed).

The results are shown in Fig. 12. The results indicate a high presence level  
15 of the protein (derived by alternative splicing from the KLK-2 gene) in the lumen-lining of prostate epithelial cells (gold color, left picture, vs. the pre-immune serum control on the right picture, where no gold color is detectable).



**CLAIMS:**

1. An isolated nucleic acid sequence selected from the group consisting of:
  - (i) the nucleic acid sequence depicted in any one of SEQ ID NO: 1 to SEQ ID NO:6.
  - 5 (ii) nucleic acid sequences having at least 70% identity with the sequence of (i); and
  - (iii) fragments of (i) or (ii) of at least 20 b.p provided that said fragment contains a sequence which is not present in the original sequence of PSA from which the sequences of (i) have been varied by alternative splicing.
- 10 2. A nucleic acid sequence according to Claim 1(ii) wherein the nucleic acid sequences have at least 80% identity with the sequence of Claim 1(i).
3. A nucleic acid sequence according to Claim 2, wherein the nucleic acid sequences have at least 90% identity.
4. An isolated nucleic acid sequence complementary to the nucleic acid  
15 sequence of Claim 1.
5. An amino acid sequence selected from the group consisting of:
  - (i) an amino acid sequence coded by the isolated nucleic acid sequence of Claim 1;
  - (ii) fragments of the amino acid sequence of (i) having at least 10 amino  
20 acids;
  - (iii) analogues of the amino acid sequences of (i) or (ii) in which one or more amino acids has been added, deleted, replaced or chemically modified without substantially altering the biological activity of the parent amino acid sequence.
- 25 6. An amino acid sequence according to Claim 5, as depicted in any one of SEQ ID NO:7 to SEQ ID NO:12.
7. An isolated nucleic acid sequence coding for the amino acid sequence of Claim 5 or 6.
8. A purified antibody which binds specifically to the amino acid sequence of  
30 Claim 5 or 6.

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9. A purified antibody which binds to an amino acid sequence present in any one of the amino acid sequence of Claim 5 or 6 and which is not present in the original PSA sequence.
10. A purified antibody according to Claim 8 or 9, conjugated to a cytotoxic  
5 compound.
11. An expression vector comprising the nucleic acid sequences of Claim 1 or 7 and control elements for the expression of the nucleic acid sequence in a suitable host.
12. An expression vector comprising the nucleic acid sequence of Claim 4, and  
10 control elements for the expression of the nucleic acid sequence in a suitable host.
13. A host cell transfected by the expression vector of Claim 11 or 12.
14. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and as an active ingredient an agent selected from the group consisting of:
- (i) the expression vector of Claim 11; and
  - 15 (ii) the amino acid sequence of Claim 5 or 6.
15. A pharmaceutical composition according to Claim 14, for treatment of diseases which can be ameliorated or cured by raising the level of the prostate specific antigen like (PSA variant) product.
16. A pharmaceutical composition comprising a pharmaceutically acceptable  
20 carrier and as an active ingredient an agent selected from the group consisting of:
- (i) the nucleic acid sequence of Claim 4;
  - (ii) the expression vector of Claim 12; and
  - (iii) the purified antibody of Claim 8 or 9.
17. A pharmaceutical composition according to Claim 16, for treatment of  
25 diseases which can be ameliorated or cured by decreasing the level of the PSA variant product.
18. A pharmaceutical composition for selective destruction of cells expressing membrane-bound PSA variant product comprising a pharmaceutically acceptable carrier and as an active ingredient an antibody of Claim 10.

19. A method for detecting an PSA variant nucleic acid sequence in a biological sample, comprising the steps of:

(a) hybridizing to nucleic acid material of said biological sample a nucleic acid sequence of Claim 1 or 4; and

5 (b) detecting hybridization complexes;

wherein the presence of said hybridization complex correlates with the presence of an PSA variant nucleic acid sequence in the said biological sample.

20. A method for determining the level of nucleic acid sequences of PSA variants in a biological sample comprising the steps of:

10 (a) hybridizing to nucleic acid material of said biological sample any one of the nucleic acid sequences of claim 1 or 4; and

(b) determining the amount of hybridization complexes and normalizing said amount to provide the level of the PSA variant nucleic acid sequences in the sample.

15 21. A method for determining the ratio between the level of the nucleic acid sequence of a PSA variant in a first biological sample and the level of the original PSA sequence from which the variant has been varied by alternative splicing, in a second biological sample comprising:

(a) determining the level of the PSA variant nucleic acid sequence in the  
20 first biological sample according to the method of Claim 20;

(b) determining the level of the PSA original sequence in the second biological sample ; and

(c) comprising the levels obtained in (a) and (b) to give said ratio.

25 22. A method according to Claim 21, wherein said first and said second biological samples are the same sample.

23. A method according to Claim 19 to 22, wherein the nucleic acid material of said biological sample are mRNA transcripts.

24. A method according to Claim 19 to 23, where the nucleic acid sequence is present in a nucleic acid chip.

– 50 –

25. A method according to any of Claims 19 to 24 for detection of the presence of prostate cancer, detection of predisposition to prostate cancer or evaluation of the malignancy of prostate cancer.

26. A method for identifying candidate compounds capable of binding to the  
5 PSA variant product and modulating its activity the method comprising:

- (i) providing a protein or polypeptide comprising an amino acid sequence substantially as depicted in any one of SEQ ID NO: 7 to SEQ ID NO: 12, or a fragment of such a sequence;
- (ii) contacting a candidate compound with said amino acid sequence;
- 10 (iii) determining the effect of said candidate compound on the biological activity of said protein or polypeptide and selecting those compounds which show a significant effect on said biological activity.

27. A method according to Claim 26, wherein the compound is an activator and the measured effect is increase in the biological activity.

15 28. A method according to Claim 26, wherein the compound is an deactivator and the effect is decrease in the biological activity.

29. An activator of the amino acid sequence of Claim 5 or 6.

30. An deactivator of the amino acid sequence of Claims 5 or 6.

31. A method for detecting PSA variant-product in a biological sample,  
20 comprising the steps of:

- (a) contacting with said biological sample the antibody of Claim 8 or 9, thereby forming an antibody-antigen complex; and

- (b) detecting said antibody-antigen complex  
wherein the presence of said antibody-antigen complex correlates with the  
25 presence of PSA variant product in said biological sample.

32. A method for determining the level of amino acid sequences of PSA variants according to claim 5 or 6 in a biological sample comprising the steps of:

- (a) contacting with said biological sample the antibody of Claim 8 or 9, thereby forming an antibody-antigen complex; and

(b) detecting said the amount of said antibody-antigen complex and normalizing said amount to provide the level of the amino acid sequence in the sample.

33. A method for determining the ratio between the level of any one of the  
5 amino acid sequences of claims 5 or 6 of the PSA variant present in a first biological sample and the level of the original PSA sequence from which the variant has been varied by alternative splicing, in a second biological sample comprising:

(a) determining the level of the PSA variant amino acid sequence in the  
10 first biological sample according to the method of Claim 32;

(b) determining the level of the PSA original sequence in the second biological sample ; and

(c) comparing the levels obtained in (a) and (b) to give said ratio.

34. A method according to Claim 33, wherein said first and said second  
15 biological samples are the same sample.

35. A method according to any one of Claims 31 to 34 for detecting the presence of prostate cancer or detecting pre-disposition to prostate cancer, or for detection of the malignancy of prostate cancer.

1/25

1 AAGCTTCTAG TTTTCTTTTC CCGGTGACAT CGTGGAAGC ACTAGCATCT  
51 CTAAGCAATG ATCTGTGACA ATATTCACAG TGTAATGCCA TCCAGGGAAC  
101 TCAACTGAGC CTTGATGTCC AGAGATTTTT GTGTTTTTTT CTGAGACTGA  
151 GTCTCGCTCT GTGCCAGGCT GGAGTGCAGT GGTGCAACCT TGGCTCACTG  
201 CAAGCTCCGC CTCCTGGGTT CACGCCATTC TCCTGCCTCA GCCTCCTGAG  
251 TAGCTGGGAC TACAGGCACC CGCCACCACG CCTGGCTAAT TTTTTTGTAT  
301 TTTTAGTAGA GATGGGGTTT CACTGTGTTA GCCAGGATGG TCTCAGTCTC  
351 CTGACCTCGT GATCTGCCCA CCTTGGCCTC CCAAAGTGCT GGGATGACAG  
401 GCGTGAGCCA CCGCGCCTGG CCGATATCCA GAGATTTTTT GGGGGGCTCC  
451 ATCACACAGA CATGTTGACT GTCTTCATGG TTGACTTTTA GTATCCAGCC  
501 CCTCTAGAAA TCTAGCTGAT ATAGTGTGGC TCAAAACCTT CAGCACAAAT  
551 CACACCGTTA GACTATCTGG TGTGGCCCAA ACCTTCAGGT GAACAAAGGG  
601 ACTCTAATCT GGCAGGATAT TCCAAAGCAT TAGAGATGAC CTCTTGCAAA  
651 GAAAAAGAAA TGGAAAAGAA AAAGAAAGAA AGGAAAAAAA AAAAAAAAAA  
701 GAGATGACCT CTCAGGCTCT GAGGGGAAAC GCCTGAGGTC TTTGAGCAAG  
751 GTCAGTCCTC TGTGTCACAG TCTCCCTCAC AGGGTCATTG TGACGATCAA  
801 ATGTGGTCAC GTGTATGAGG CACCAGCACA TGCCTGGCTC TGGGGAGTGC  
851 CGTGTAAGTG TATGCTTGCA CTGCTGAATG CTTGGGATGT GTCAGGGATT  
901 ATCTTCAGCA CTTACAGATG CTCATCTCAT CCTCACAGCA TCACTATGGG  
951 ATGGGTATTA CTGGCCTCAT TTGATGGAGA AAGTGGCTGT GGCTCAGAAA  
1001 GGGGGGACCA CTAGACCAGG GACACTCTGG ATGCTGGGGA CTCCAGAGAC  
1051 CATGACCACT CACCAACTGC AGAGAAATTA ATTGTGGCCT GATGTCCCTG  
1101 TCCTGGAGAG GGTGGAGGTG GACCTTCACT AACCTCCTAC CTTGACCCTC

Fig. 1

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1151 TCTTTTAGGG CTCTTTCTGA CCTCCACCAT GGTACTAGGA CCCCATTTGTA  
1201 TTCTGTACCC TCTTGACTCT ATGACCCCCA CTGCCCCTG CATCCAGCTG  
1251 GGTCCCCTCC TATCTCTATT CCCAGCTGGC CAGTGCAGTC TCAGTGCCCA  
1301 CCTGTTTGTC AGTAACTCTG AAGGGGCTGA CATTTTACTG ACTTGCAAAC  
1351 AAATAAGCTA ACTTTCCAGA GTTTTGTGAA TGCTGGCAGA GTCCATGAGA  
1401 CTCCTGAGTC AGAGGCAAAG GCTTTTACTG CTCACAGCTT AGCAGACAGC  
1451 ATGAGGTTCA TGTTACATT AGTACACCTT GCCCCCCCCA AATCTTGTAG  
1501 GGTGACCAGA GCAGTCTAGG TGGATGCTGT GCAGAAGGGG TTTGTGCCAC  
1551 TGGTGAGAAA CCTGAGATTA GGAATCCTCA ATCTTATACT GGGACAACCTT  
1601 GCAAACCTGC TCAGCCTTTG TCTCTGATGA AGATATTATC TTCATGATCT  
1651 TGGATTGAAA ACAGACCTAC TCTGGAGGAA CATATTGTAT CGATTGTCCT  
1701 TGACAGTAAA CAAATCTGTT GTAAGAGACA TTATCTTTAT TATCTAGGAC  
1751 AGTAAGCAAG CCTGGATCTG AGAGAGATAT CATCTTGCAA GGATGCCTGC  
1801 TTTACAAACA TCCTTGAAAC AACAATCCAG AAAAAAAAAAG GTGTTGCTGT  
1851 CTTTGCTCAG AAGACACACA GATACGTGAC AGAACCATGG AGAATTGCCT  
1901 CCCAACGCTG TTCAGCCAGA GCCTTCCACC CTTGTCTGCA GGACAGTCTC  
1951 AACGTTCCAC CATTAAATAC TTCTTCTATC ACATCCTGCT TCTTTATGCC  
2001 TAACCAAGGT TCTAGGTCCC GATCGACTGT GTCTGGCAGC ACTCCACTGC  
2051 CAAACCCAGA ATAAGGCAGC GCTCAGGATC CCGAAGGGGC ATGGCTGGGG  
2101 ATCAGAACTT CTGGGTTTGA GTGAGGAGTG GGTCCACCCT CTTGAATTTT  
2151 AAAGGAGGAA GAGGCTGGAT GTGAAGGTAC TGGGGGAGGG AAAGTGTGAG  
2201 TTCCGAACTC TTAGGTCAAT GAGGGAGGAG ACTGGTAAGG TCCCAGCTCC  
2251 CGAGGTACTG ATGTGGGAAT GGCCTAAGAA TCTCATATCC TCAGGAAGAA  
2301 GGTGCTGGAA TCCTGAGGGG TAGAGTTCTG GGTATATTTG TGGCTTAAGG

Fig.1 (Cont.)

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2351 CTCTTTGGCC CCTGAAGGCA GAGGCTGGAA CCATTAGGTC CAGGGTTTGG  
2401 GGTGATAGTA ATGGGATCTC TTGATTCCCTC AAGAGTCTGA GGATCGAGGG  
2451 TTGCCCATTC TTCCATCTTG CCACCTAATC CTTACTCCAC TTGAGGGTAT  
2501 CACCAGCCCT TCTAGCTCCA TGAAGGTCCC CTGGGCAAGC ACAATCTGAG  
2551 CATGAAAGAT GCCCCAGAGG CCTTGGGTGT CATCCACTCA TCATCCAGCA  
2601 TCACACTCTG AGGGTGTGGC CAGCACCATG ACGTCATGTT GCTGTGACTA  
2651 TCCCTGCAGC GTGCCTCTCC AGCCACCTGC CAACCGTAGA GCTGCCCATC  
2701 CTCCTCTGGT GGGAGTGGCC TGCATGGTGC CAGGCTGAGG CCTAGTGTCA  
2751 GACAGGGAGC CTGGAATCAT AGGGATCCAG GACTCAAAAG TGCTAGAGAA  
2801 TGGCCATATG TCACCATCCA TGAAATCTCA AGGGCTTCTG GGTGGAGGGC  
2851 ACAGGGACCT GAACCTATGG TTTCCCAAGT CTATTGCTCT CCCAAGTGAG  
2901 TCTCCCAGAT ACGAGGCACT GTGCCAGCAT CAGCCTTATC TCCACCACAT  
2951 CTTGTAAAAG GACTACCCAG GGCCCTGATG AACACCATGG TGTGTACAGG  
3001 AGTAGGGGGT GGAGGCACGG ACTCCTGTGA GGTACAGCC AAGGGAGCAT  
3051 CATCATGGGT GGGGAGGAGG CAATGGACAG GCTTGAGAAC GGGGATGTGG  
3101 TTGTATTTGG TTTTCTTTGG TTAGATAAAG TGCTGGGTAT AGGATTGAGA  
3151 GTGGAGTATG AAGACCAGTT AGGATGGAGG ATCAGATTGG AGTTGGGTTA  
3201 GATAAAGTGC TGGGTATAGG ATTGAGAGTG GAGTATGAAG ACCAGTTAGG  
3251 ATGGAGGATC AGATTGGAGT TGGGTTAGAG ATGGGGTAAA ATTGTGCTCC  
3301 GGATGAGTTT GGGATTGACA CTGTGGAGGT GGTTTGGGAT GGCATGGCTT  
3351 TGGGATGGAA ATAGATTTGT TTTGATGTTG GCTCAGACAT CCTTGGGGAT  
3401 TGAAGTGGGG ATGAAGCTGG GTTTGATTTT GGAGGTAGAA GACGTGGAAG  
3451 TAGCTGTCAG ATTTGACAGT GGCCATGAGT TTTGTTTGAT GGGGAATCAA

Fig. 1 (Cont.)



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3501 ACAATGGGGG AAGACATAAG GGTGGGCTTG TTAGGTTAAG TTGCGTTGGG  
3551 TTGATGGGGT CGGGGCTGTG TATAATGCAG TTGGATTGGT TTGTATTAAA  
3601 TTGGGTTGGG TCAGGTTTTG GTTGAGGATG AGTTGAGGAT ATGCTTGGGG  
3651 ACACCGGATC CATGAGGTTC TCACTGGAGT GGAGACAAAC TTCCTTTCCA  
3701 GGATGAATCC AGGGAAGCCT TAATTCACGT GTAGGGGAGG TCAGGCCACT  
3751 GGCTAAGTAT ATCCTTCCAC TCCAGCTCTA AGATGGTCTT AAATTGTGAT  
3801 TATCTATATC CACTTCTGTC TCCCTCACTG TGCTTGGAGT TTACCTGATC  
3851 ACTCAACTAG AAACAGGGGA AGATTTTATC AAATTCTTTT TTTTTTTTTT  
3901 TTTTTTTTGA GACAGAGTCT CACTCTGTTG CCCAGGCTGG AGTGCAGTGG  
3951 CGCAGTCTCG GCTCACTGCA ACCTCTGCCT CCCAGGTTCA AGTGATTCTC  
4001 CTGCCTCAGC CTCCTGAGTT GCTGGGATTA CAGGCATGCA GCACCATGCC  
4051 CAGCTAATTT TTGTATTTTT AGTAGAGATG GGGTTTCACC AATGTTTGCC  
4101 AGGCTGGCCT CGAACTCCTG ACCTGGTGAT CCACCTGCCT CAGCCTCCCA  
4151 AAGTGCTGGG ATTACAGGCG TCAGCCACCG CGCCCAGCCA CTTTTGTCAA  
4201 ATTCTTGAGA CACAGCTCGG GCTGGATCAA GTGAGCTACT CTGGTTTTAT  
4251 TGAACAGCTG AAATAACCAA CTTTTTGGAA ATTGATGAAA TCTTACGGAG  
4301 TTAACAGTGG AGGTACCAGG GCTCTTAAGA GTTCCCGATT CTCTTCTGAG  
4351 ACTACAAATT GTGATTTTGC ATGCCACCTT AATCTTTTTT TTTTTTTTTT  
4401 TAAATCGAGG TTTCAGTCTC ATTCTATTTC CCAGGCTGGA GTTCAATAGC  
4451 GTGATCACAG CTCACTGTAG CCTTGAATC CTGGCCTTAA GAGATTCTCC  
4501 TGCTTCGGTC TCCAATAGC TAAGACTACA GTAGTCCACC ACCATATCCA  
4551 GATAATTTTT AAATTTTTTG GGGGGCCGGG CACAGTGGCT CACGCCTGTA  
4601 ATCCCAACAC CATGGGAGGC TGAGATGGGT GGATCACGAG GTCAGGAGTT

Fig.1 (Cont.)

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4651 TGAGACCAGC CTGACCAACA TGGTGAAACT CTGTCTCTAC TAAAAAAAAA  
4701 AAAAATAGAA AAATTAGCCG GCGTGGGTGG CACACGGCAC CTGTAATCCC  
4751 AGCTACTGAG GAGGCTGAGG CAGGAGAATC ACTTGAACCC AGAAGGCAGA  
4801 GGTTGCAATG AGCCGAGATT GCGCCACTGC ACTCCAGCCT GGGTGACAGA  
4851 GTGAGACTCT GTCTCAAAAA AAAAAAATTT TTTTTTTTTT TTTGTAGAGA  
4901 TGGATCTTGC TTTGTTTCTC TGGTTGGCCT TGAATCCTG GCTTCAAGTG  
4951 ATCCTCCTAC CTTGGCCTCG GAAAGTGTTG GGATTACAGG CGTGAGCCAC  
5001 CATGACTGAC CTGTCGTTAA TCTTGAGGTA CATAAACCTG GCTCCTAAG  
5051 GCTAAAGGCT AAATATTTGT TGGAGAAGGG GCATTGGATT TTGCATGAGG  
5101 ATGATTCTGA CCTGGGAGGG CAGGTCAGCA GGCATCTCTG TTGCACAGAT  
5151 AGAGTGTACA GGTCTGGAGA ACAAGGAGTG GGGGGTTATT GGAATTCCAC  
5201 ATTGTTTGCT GCACGTTGGA TTTTGAAATG CTAGGGAAct TTGGGAGACT  
5251 CATATTTCTG GGCTAGAGGA TCTGTGGACC ACAAGATCTT TTTATGATGA  
5301 CAGTAGCAAT GTATCTGTGG AGCTGGATTC TGGGTGGGA GTGCAAGGAA  
5351 AAGAATGTAC TAAATGCCAA GACATCTATT TCAGGAGCAT GAGGAATAAA  
5401 AGTTCTAGTT TCTGGTCTCA GAGTGGTGCA GGGATCAGGG AGTCTCACAA  
5451 TCTCCTGAGT GCTGGTGTCT TAGGGCACAC TGGGTCTTGG AGTGCAAAGG  
5501 ATCTAGGCAC GTGAGGCTTT GTATGAAGAA TCGGGGATCG TACCCACCCC  
5551 CTGTTTCTGT TTCATCCTGG GCATGTCTCC TCTGCCTTTG TCCCCTAGAT  
5601 GAAGTCTCCA TGAGCTACAG GGCCTGGTGC ATCCAGGGTG ATCTAGTAAT  
5651 TGCAGAACAG CAAGTGCTAG CTCTCCCTCC CCTTCCACAG CTCTGGGTGT  
5701 GGGAGGGGGT TGTCCAGCCT CCAGCAGCAT GGGGAGGGCC TTGGTCAGCC  
5751 TCTGGGTGCC AGCAGGGCAG GGGCGGAGTC CTGGGGAATG AAGGTTTTAT  
5801 AGGGCTCCTG GGGGAGGCTC CCCAGCCCCA AGCTTACCAC CTGCACCCGG

Fig.1 (Cont.)

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5851 AGAGCTGTGT CACCATGTGG GTCCCGGTTG TCTTCCTCAC CCTGTCCGTG  
5901 ACGTGGATTG GTGAGAGGGG CCATGGTTGG GGGGATGCAG GAGAGGGAGC  
5951 CAGCCCTGAC TGTCAAGCTG AGGCTCTTTC CCCCCAACC CAGCACCCCA  
6001 GCCCAGACAG GGAGCTGGGC TCTTTTCTGT CTCTCCCAGC CCCACTCCAA  
6051 GCCCATACCC CCAGCCCCTC CATATTGCAA CAGTCCTCAC TCCCACACCA  
6101 GGTCCCCGCT CCCTCCCCTT TACCCAGAA CTTTCTCCCC ATTTGCCAG  
6151 CCAGCTCCCT GCTCCCAGCT GCTTTACTAA AGGGGAAGTT CCTGGGCATC  
6201 TCCGTGTTTC TCTTTGTGGG GCTCAAACC TCCAAGGACC TCTCTCAATG  
6251 CCATTGGTTC CTTGGACCGT ATCACTGGTC CACCTCCTGA GCCCTCAAT  
6301 CCTATCACAG TCTACTGACT TTTCCATTCA GCTGTGAGTG CCCAACCTA  
6351 TCCCAGAGAC CTTGATGCTT GGCCTCCCAA TCTTGCCCTA GGATACCCAG  
6401 ATGCCAACCA GACACCTCCT TCTTCCTAGC CAGGCTATCT GGCTGAGACA  
6451 ACAATGGGT CCCTCAGTCT GGCAATGGGA CTCTGAGAAC TCCTCATTC  
6501 CTGACTCTTA GCCCCAGACT CTTCATTCAG TGGCCACAT TTTCTTAGG  
6551 AAAACATGA GCATCCCCAG CCACAACCTGC CAGCTCTCTG ATTCCCCAAA  
6601 TCTGCATCCT TTTCAAACC TAAAAACAAA AAGAAAAACA AATAAAACAA  
6651 AACCAACTCA GACCAGAACT GTTTTCTCAA CCTGGGACTT CCTAAACTTT  
6701 CCAAACCTT CCTCTTCCAG CAACTGAACC TCCCGATAAG GCACTTATCC  
6751 CTGGTTCCTA GCACCGCTTA TCCCCTCAGA ATCCACAACCT TGTACCAAGT  
6801 TTCCCTTCTC CCAGTCCAAG ACCCCAAATC ACCACAAAGG ACCCAATCCC  
6851 CAGACTCAAG ATATGGTCTG GGGCTGTCTT GTGTCTCCTA CCCTGATCCC  
6901 TGGGTTCAAC TCTGTCCCAG AGCATGAAGC CTCTCCACCA GCACCAGCCA  
6951 CCAACCTGCA AACCTAGGGA AGATTGACAG AATTCCCAGC CTTTCCCAGC

Fig.1(Cont.)

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7001 TCCCCCTGCC CATGTCCCAG GACTCCCAGC CTTGGTTCTC TGCCCCCGTG  
7051 TCTTTTCAAA CCCACATCCT AAATCCATCT CCTATCCGAG TCCCCCAGTT  
7101 CCTCCTGTCA ACCCTGATTC CCCTGATCTA GCACCCCCTC TGCAGGTGCT  
7151 GCACCCCTCA TCCTGTCTCG GATTGTGGGA GGCTGGGAGT GCGAGAAGCA  
7201 TTCCCAACCC TGGCAGGTGC TTGTGGCCTC TCGTGGCAGG GCAGTCTGCG  
7251 GCGGTGTTCT GGTGCACCCC CAGTGGGTCC TCACAGCTGC CCACTGCATC  
7301 AGGAAGTGAG TAGGGGCCTG GGGTCTGGGG AGCAGGTGTC TGTGTCCAGA  
7351 GGAATAACAG CTGGGCATTT TCCCCAGGAT AACCTCTAAG GCCAGCCTTG  
7401 GGACTGGGGG AGAGAGGGAA AGTTCTGGTT CAGGTCACAT GGGGAGGCAG  
7451 GGTTGGGGCT GGACCACCCT CCCCATGGCT GCCTGGGTCT CCATCTGTGT  
7501 TCCTCTATGT CTCTTTGTGT CGCTTTCATT ATGTCTCTTG GTAAGTGGCT  
7551 TCGGTTGTGT CTCTCCGTGT GACTATTTTG TTCTCTCTCT CCCTCTCTTC  
7601 TCTGTCTTCA GTCTCCATAT CTCCCCCTCT CTCTGTCCTT CTCTGGTCCC  
7651 TCTCTAGCCA GTGTGTCTCA CCCTGTATCT CTCTGCCAGG CTCTGTCTCT  
7701 CGGTCTCTGT CTCACCTGTG CCTTCTCCCT ACTGAGCACA CGCATGGGAT  
7751 GGGCCTGGGG GGACCCTGAG AAAAGGAAGG GCTTTGGCTG GGCGCGGTGG  
7801 CTCACACCTG TAATCCCAGC ACTTTGGGAG GCCAAGGCAG GTAGATCACC  
7851 TGAGGTCAGG AGTTCGAGAC CAGCCTGGCC AACTGGTGAA ACCCCATCTC  
7901 TACTAAAAAT ACAAAAAATT AGCCAGGCGT GGTCGGCGCA TGCCTGTAGT  
7951 CCCAGCTACT CAGGAGGCTG AGGGAGGAGA ATTGCTTGAA CCTGGGAGGT  
8001 GGAGGTTGCA GTGAGCCGAG ACGTGCCACT GCACTCCAGC CTGGGTGACA  
8051 GAGTGAGACT CCGCCTCAAA AAAAAAAAAA AAAAAAAGA AAAGAAAAGA  
8101 AAAGAAAAGG AAGTGTTTTA TCCCTGATGT GTGTGGGTAT GAGGGTATGA  
8151 GAGGGCCCCT CTCACCTCAT TCCTTCTCCA GGACATCCCT CCACTCTTGG  
8201 GAGACACAGA GAAGGGCTGG TTCAGCTGGA GCTGGGAGGG GCAATTGAGG

Fig.1 (Cont.)

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8251 GAGGAGGAAG GAGAAGGGGG AAGGAAAACA GGGTATGGGG GAAAGGACCC  
8301 TGGGGAGCGA AGTGGAGGAT ACAACCTTGG GCCTGCAGGC CAGGCTACCT  
8351 ACCCACTTGG AAACCCACGC CAAAGCCGCA TCTACAGCTG AGCCACTCTG  
8401 AGGCCTCCCC TCCCCAGCGG TCCCCACTCA GCTCCAAAGT CTCTCTCCCT  
8451 TTTCTCTCCC AACTCTATC ATCCCCCGGA TTCCTCTCTA CTTGGTTCTC  
8501 ATTCTTCCTT TGA CTTCCTG CTTCCCTTTC TCATTCATCT GTTTCTCACT  
8551 TTCTGCCTGG TTTTGTTCTT CTCTCTCTCT TTCTCTGGCC CATGTCTGTT  
8601 TCTCTATGTT TCTGTCTTTT CTTTCTCATC CTGTGTATTT TCGGCTCACC  
8651 TTGTTTGTCA CTGTTCTCCC CTCTGCCCTT TCATTCTCTC TGTCTTTTA  
8701 CCCTCTTCCT TTTTCCCTTG GTTTCTCTCA GTTTCTGTAT CTGCCCTTCA  
8751 CCCTCTCACA CTGCTGTTTC CCAACTCGTT GTCTGTATTT TTGGCCTGAA  
8801 CATGTGTCTT CCCC AACCCT GTGTTTTTCT CACTGTTTCT TTTTCTCTTT  
8851 TGGAGCCTCC TCCTTGCTCC TCTGTCCCTT CTCTCTTTCC TTATCATCCT  
8901 CGCTCCTCAT TCCTGCGTCT GCTTCCTCCC CAGCAAAGC GTGATCTTGC  
8951 TGGGTCGGCA CAGCCTGTTT CATCCTGAAG ACACAGGCCA GGTATTTTCA  
9001 GTCAGCCACA GCTTCCCACA CCCGCTCTAC GATATGAGCC TCCTGAAGAA  
9051 TCGATTCCTC AGGCCAGGTG ATGACTCCAG CCACGACCTC ATGCTGCTCC  
9101 GCCTGTCAGA GCCTGCCGAG CTCACGGATG CTGTGAAGGT CATGGACCTG  
9151 CCCACCCAGG AGCCAGCACT GGGGACCACC TGCTACGCCT CAGGCTGGGG  
9201 CAGCATTGAA CCAGAGGAGT GTACGCCTGG GCCAGATGGT GCAGCCGGGA  
9251 GCCCAGATGC CTGGGTCTGA GGGAGGAGGG GACAGGACTC CTAGGTCTGA  
9301 GGGAGGAGGG CCAAGGAACC AGGTGGGGTC CAGCCCACAA CAGTGTTTTT  
9351 TGCCTGGCCC GTAGTCTTGA CCCCAAAGAA ACTTCAGTGT GTGGACCTCC

Fig.1 (Cont.)

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9401 ATGTTATTTTC CAATGACGTG TGTGCGCAAG TTCACCCTCA GAAGGTGACC  
9451 AAGTTCATGC TGTGTGCTGG ACGCTGGACA GGGGGCAAAA GCACCTGCTC  
9501 GGTGAGTCAT CCCTACTCCC AAGATCTTGA GGGGAAAGGT GAGTGGGGAC  
9551 CTTAATTCTG GGCTGGGGTC TAGAAGCCAA CAAGCATCTG CCTCCCCTGC  
9601 TCCCCAGCTG TAGCCATGCC ACCTCCCCGT GTCTCATCTC ATTCCCTCCT  
9651 TCCCTCTTCT TTGACTCCCT CAAGGCAATA GGTTATTCTT ACAGCACAAAC  
9701 TCATCTGTTC CTGCGTTCAG CACACGGTTA CTAGGCACCT GCTATGCACC  
9751 CAGCACTGCC CTAGAGCCTG GACATAGCAG TGAACAGACA GAGAGCAGCC  
9801 CCTCCCTTCT GTAGCCCCCA AGCCAGTGAG GGGCACAGGC AGGAACAGGG  
9851 ACCACAACAC AGAAAAGCTG GAGGGTGTCA GGAGGTGATC AGGCTCTCGG  
9901 GGAGGGAGAA GGGGTGGGGA GTGTGACTGG GAGGAGACAT CCTGCAGAAG  
9951 GCGGGAGTGA GCAAACACCT GCCGCAGGGG AGGGGAGGGC CTGCGGCACC  
10001 TGGGGGAGCA GAGGGAACAG CATCTGGCCA GCCTTGGGAG GAGGGGCCTA  
10051 GAGGGCGTCA GGAGCAGAGA GGAGGTTGCC TGGCTGGAGT GAAGGATCGG  
10101 GGCAGGGTGC GAGAGGGAAG AAGGACCCCT CCTGCAGGGC CTCACCTGGG  
10151 CCACAGGAGG ACACTGCTTT TCCTCTGAGG AGTCAGGAAC TGTGGATGGT  
10201 GCTGGACAGA AGCAGGACAG GGCCTGGCTC AGGTGTCCAG AGGCTGCCGC  
10251 TGGCCTCCCT ATGGGATCAG ACTGCAGGGA GGGAGGGCAG CAGGGATGTG  
10301 GAGGGAGTGA TGATGGGGCT GACCTGGGGG TGGCTCCAGG CATTGTCCCC  
10351 ACCTGGGCCC TTACCCAGCC TCCCTCACAG GCTCCTGGCC CTCAGTCTCT  
10401 CCCCTCCACT CCATTCTCCA CCTACCCACA GTGGGTCATT CTGATCACCG  
10451 AACTGACCAT GCCAGCCCTG CCGATGGTCC TCCATGGCTC CCTAGTGCCC  
10501 TGGAGAGGAG GTGTCTAGTC AGAGAGTAGT CCTGGAAGGT GGCCTCTGTG  
10551 AGGAGCCACG GGGACAGCAT CCTG

Fig.1 (Cont.)

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MWVPWFELTSLVTWIGAAPLILSRIVGWGECEKHSQPWQVLVAS  
RGRAVCGGVLVHPQWVLTAAHCIRKCKSVILLGRHSLFHPEDTGQVFQVSHSFPHPLY  
DMSLLKNRFLRPGDDSSHDLMLLRLSEPAELTDAVKVMDLPTQEPALGTTTCYASGWGS  
IEPEEFLTPKKLQCVDLHVISNDVCAQVHPQKVTKFMLCAGRWTGGKSTCSGDSGGPL  
VCGVLQGITSWGSEPCALPERPSLYTKVHYRKWKIKDTIVANP

Fig. 2

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```
1      50
psal_1  MKNRGSYPPP VSVSSWACLL CLCPLDEVSM SYRAWCIQGD LVIAEQQVLA
psal_2  MKNRGSYPPP VSVSSWACLL CLCPLDEVSM SYRAWCIQGD LVIAEQQVLA
psal_0  ~~~~~~ ~~~~~~ ~~~~~~ ~~~~~~ ~~~~~~
humpsantig MKNRGSYPPP VSVSSWACLL CLCPLDEVSM SYRAWCIQGD LVIAEQQVLA
psal_5  MKNRGSYPPP VSVSSWACLL CLCPLDEVSM SYRAWCIQGD LVIAEQQVLA
psal_6  MKNRGSYPPP VSVSSWACLL CLCPLDEVSM SYRAWCIQGD LVIAEQQVLA

51      100
psal_1  LPPLPQLWVW EGVVQPPAAW GGPWSASGCQ QGRGGVLGNE GFIGLLGEAP
psal_2  LPPLPQLWVW EGVVQPPAAW GGPWSASGCQ QGRGGVLGNE GFIGLLGEAP
psal_0  ~~~~~~ ~~~~~~ ~~~~~~ ~~~~~~ ~~~~~~
humpsantig LPPLPQLWVW EGVVQPPAAW GGPWSASGCQ QGRGGVLGNE GFIGLLGEAP
psal_5  LPPLPQLWVW EGVVQPPAAW GGPWSASGCQ QGRGGVLGNE GFIGLLGEAP
psal_6  LPPLPQLWVW EGVVQPPAAW GGPWSASGCQ QGRGGVLGNE GFIGLLGEAP

101     150
psal_1  QPQAYHLHPE SCVTMWVPVV FLTLSVTWIG ERGHGWGDAG EGASPDCCQAE
psal_2  QPQAYHLHPE SCVTMWVPVV FLTLSVTWIG ERGHGWGDAG EGASPDCCQAE
psal_0  ~~~~~~ ~~~~~~ ~~~~~~ ~~~~~~ ~~~~~~
humpsantig QPQAYHLHPE SCVTMWVPVV FLTLSVTWIG ERGHGWGDAG EGASPDCCQAE
psal_5  QPQAYHLHPE SCVTMWVPVV FLTLSVTWIG ERGHGWGDAG EGASPDCCQAE
psal_6  QPQAYHLHPE SCVTMWVPVV FLTLSVTWIG ERGHGWGDAG EGASPDCCQAE
```

Fig. 3



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```

151      psal_1  ALSPPTQHPS PDRELGSFLS LPAPLQ.... ...AHTPSPS ILQQSSSLPHQ 200
      psal_2  ALSPPTQHPS PDRELGSFLS LPAPLQ.... ...AHTPSPS ILQQSSSLPHQ
      psal_0  ALSPPTQHPS PDRELGSFLS LPAPLQ.... ...AHTPSPS ILQQSSSLPHQ
      humpsantig  ALSPPTQHPS PDRELGSFLS LPAPLQ.... ...AHTPSPS ILQQSSSLPHQ
      psal_5  ALSPPTQHPS PDRELGSFLS LPAPLQLPAP SCL~~~~~
      psal_6  .....
201      psal_1  VPAPSHLPQN FLPIAQAPAC SQLLY~~~~~ 250
      psal_2  VPAPSHLPQN FLPIAQAPAC SQLLY~~~~~
      psal_0  VPAPSHLPQN FLPIAQAPAC SQLLY~~~~~
      humpsantig  VPAPSHLPQN FLPI..... ...CPASS LLPAALLKCK FLGISVFLFV
      psal_5  ~~~~~
      psal_6  .....
251      psal_1  ~~~~~ 300
      psal_2  ~~~~~
      psal_0  ~~~~~
      humpsantig  GLKTSKDLSQ CHWFLGPYHW STS~~~~~
      psal_5  ~~~~~
      psal_6  ..... ...AAPLILS RIVGWECEK HSQPWQVLVA

```

Fig. 3(Cont.)

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```
301      ~~~~~ 350
psal_1  ~~~~~
psal_2  ~~~~~
psal_0  ~~~~~
humpsantig ~~~~~
psal_5  ~~~~~
psal_6  SRGRAVCGGV LVHPQWVLT AHCIRNKSVI LLGRHSLEHP EDTGQVFQVS

351      ~~~~~ 400
psal_1  ~~~~~
psal_2  ~~~~~
psal_0  ~~~~~
humpsantig ~~~~~
psal_5  ~~~~~
psal_6  HSFPHPPLYDM SLLKNRFLRP GDDSSHDLML LRLSEPAELT DAVKVDLPT

401      ~~~~~ 450
psal_6  QEPALGTTTCY ASGWGSIEPE EFLTPKKLQC VDLHVISNDV CAQVHPQKVT

451      ~~~~~ 500
psal_6  KFMLCAGRWT GGKSTCSGDS GGPLVCNGVL QGITSWGSEP CALPERPSLY

501      ~~~~~ 518
psal_6  TKVVHYRKWI KDTIVANP
```

Fig. 3(Cont.)

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```
1          50
psal_1    MKNRGSYPPP VSVSSWACLL CLCPLDEVSM SYRAWCIQGD LVIAEQQVLA
psal_2    MKNRGSYPPP VSVSSWACLL CLCPLDEVSM SYRAWCIQGD LVIAEQQVLA
psal_0    ~~~~~~
humpsantig MKNRGSYPPP VSVSSWACLL CLCPLDEVSM SYRAWCIQGD LVIAEQQVLA
psal_5    MKNRGSYPPP VSVSSWACLL CLCPLDEVSM SYRAWCIQGD LVIAEQQVLA
psal_6    MKNRGSYPPP VSVSSWACLL CLCPLDEVSM SYRAWCIQGD LVIAEQQVLA

51          100
psal_1    LPPLPQLWVW EGVVQPPAAW GGPWSASGCQ QGRGGVLGNE GFIGLLGEAP
psal_2    LPPLPQLWVW EGVVQPPAAW GGPWSASGCQ QGRGGVLGNE GFIGLLGEAP
psal_0    ~~~~~~
humpsantig LPPLPQLWVW EGVVQPPAAW GGPWSASGCQ QGRGGVLGNE GFIGLLGEAP
psal_5    LPPLPQLWVW EGVVQPPAAW GGPWSASGCQ QGRGGVLGNE GFIGLLGEAP
psal_6    LPPLPQLWVW EGVVQPPAAW GGPWSASGCQ QGRGGVLGNE GFIGLLGEAP

101          150
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psal_6    QPQAYHLHPE SCVTMWVPV FTLSVTWIG .....

```

Fig. 4

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      humpsantig  ALSPPTQHPS PDRELGSFSL LPAPLQ.... ...AHTPSPS ILQSSSLPHQ
      psal_5      ALSPPTQHPS PDRELGSFSL LPAPLQLPAP SCL~~~~~
      psal_6      .....
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      psal_2      VPAPSHLPQN FLPIAQAPC SALLY~~~~~
      psal_0      VPAPSHLPQN FLPIAQAPC SALLY~~~~~
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      psal_5      ~~~~~
      psal_6      .....
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      psal_2      ~~~~~
      psal_0      ~~~~~
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Fig. 4 (Cont.)

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      psal_2      ~~~~~
      psal_0      ~~~~~
      humpsantig  ~~~~~
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      psal_6      ~~~~~
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      psal_1      ~~~~~
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      humpsantig  ~~~~~
      psal_5      ~~~~~
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Fig. 4 (Cont.)

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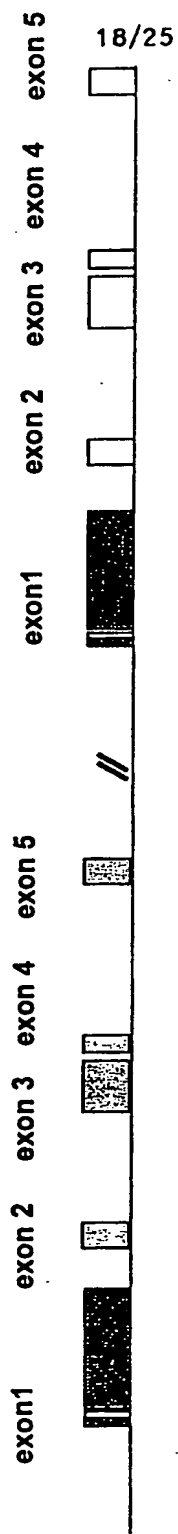
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Fig. 5

# PSALM

## KLK genomic

## PSA genomic



## Common enhancer

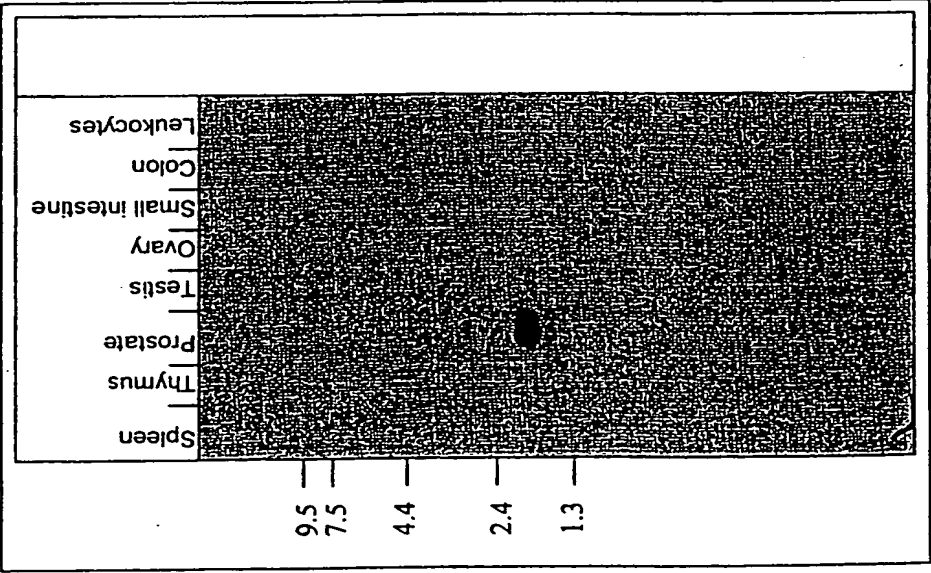
## KLK-LM

## PSA-LM

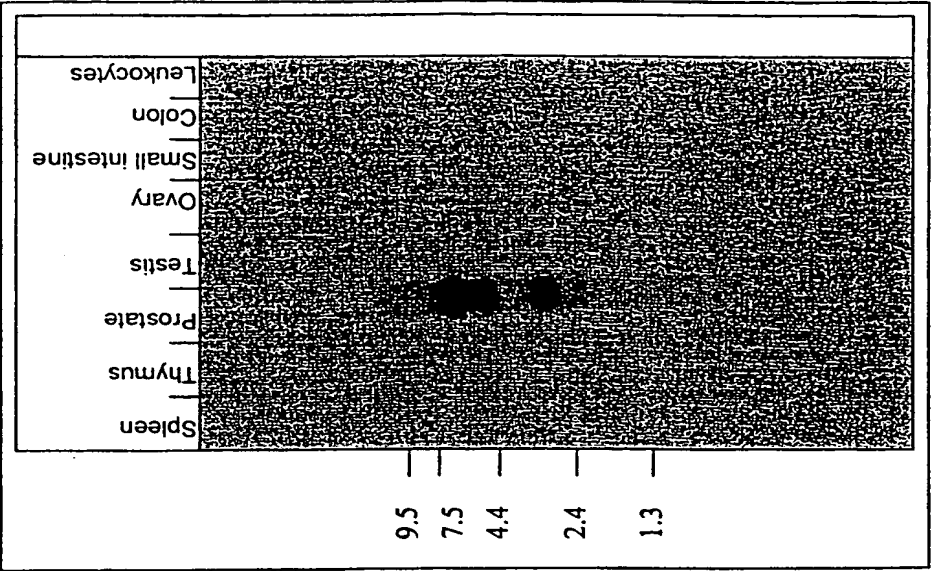
Fig. 6

Northern blot analysis:

PSA



PSALM



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Fig. 7



# Western blot analysis

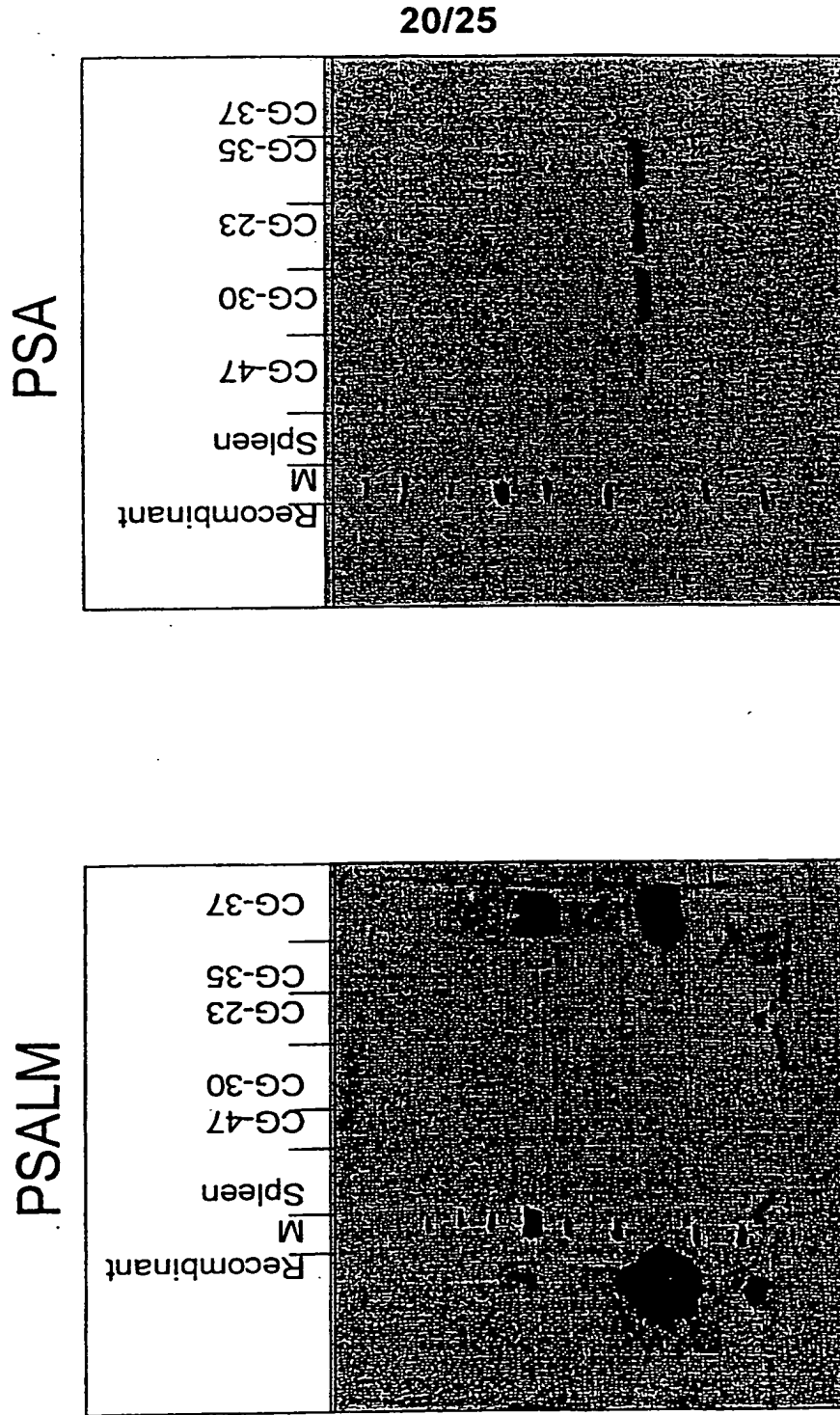
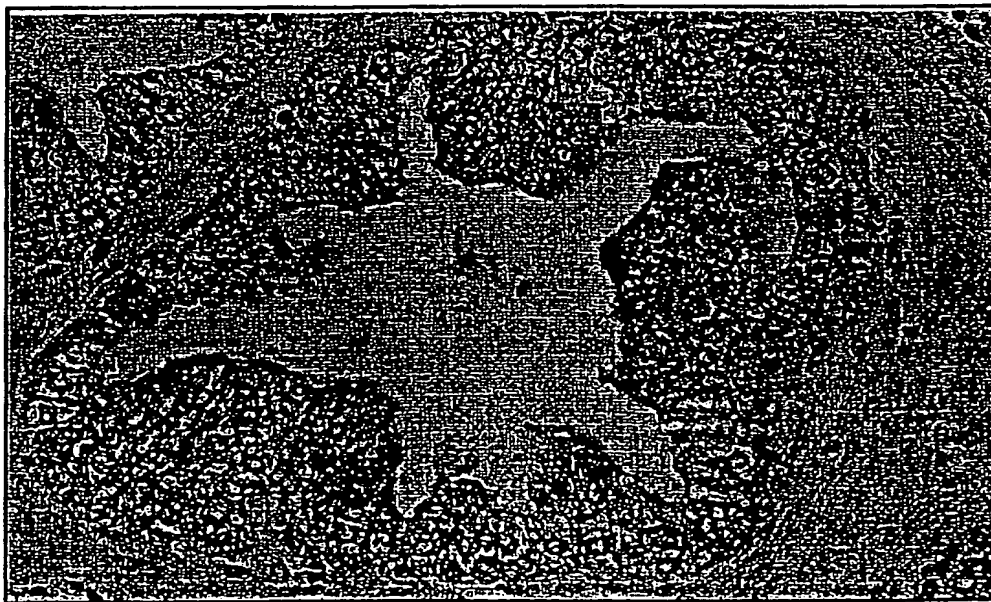


Fig. 8

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# PSALM

Pre-immune serum



Immune serum

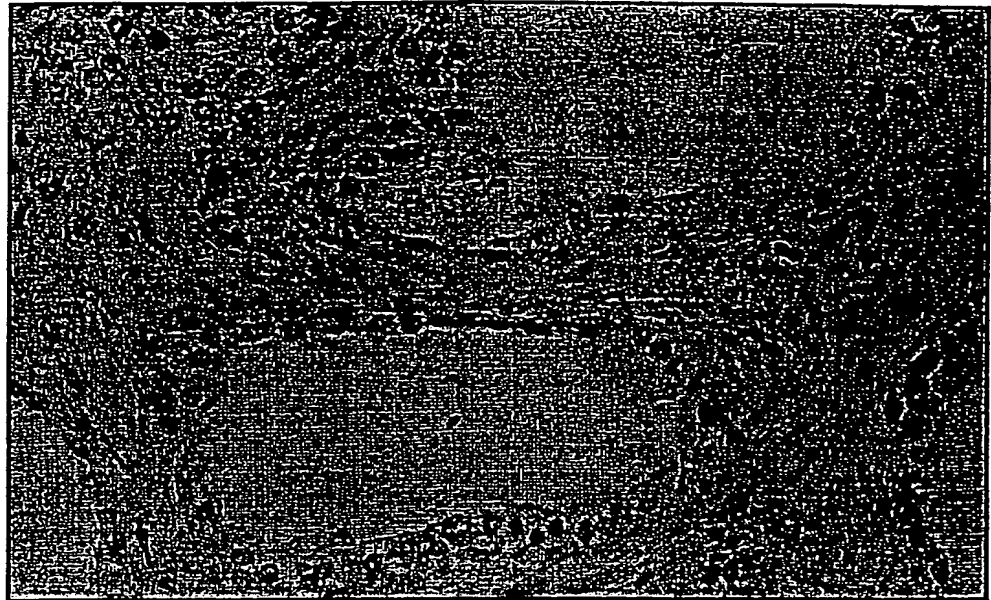


Fig. 9

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# PSALM

Anti-Sense



Sense

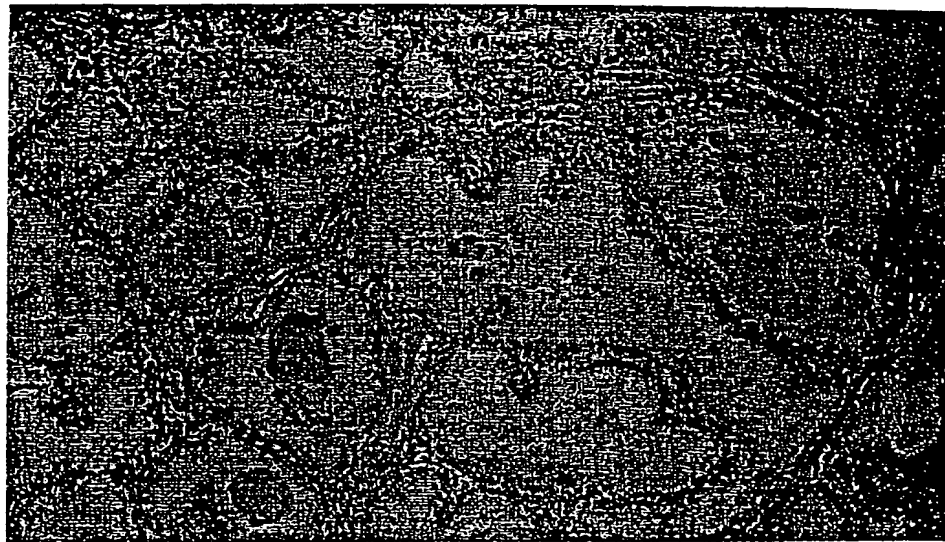


Fig. 10

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# Western blot - anti PSALM

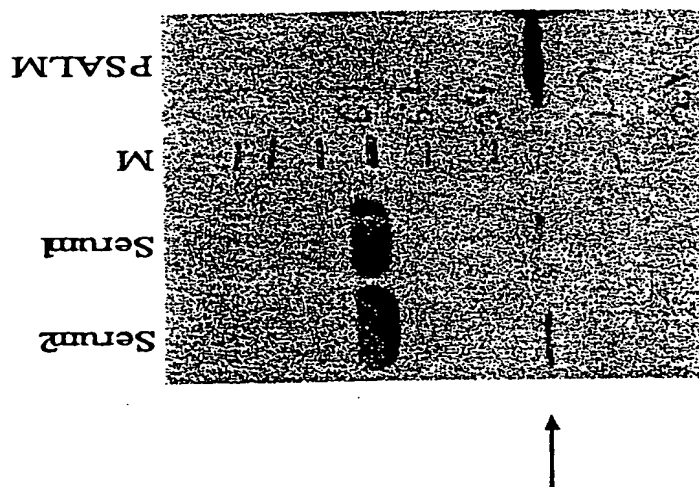
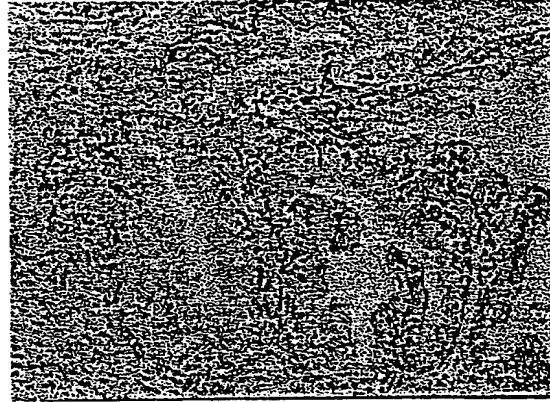


Fig. 11

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KLM

Immune serum



Pre-immune serum

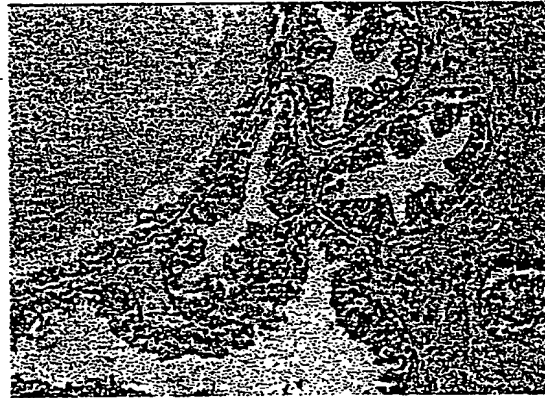


Fig. 12

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Western blot - anti KLM

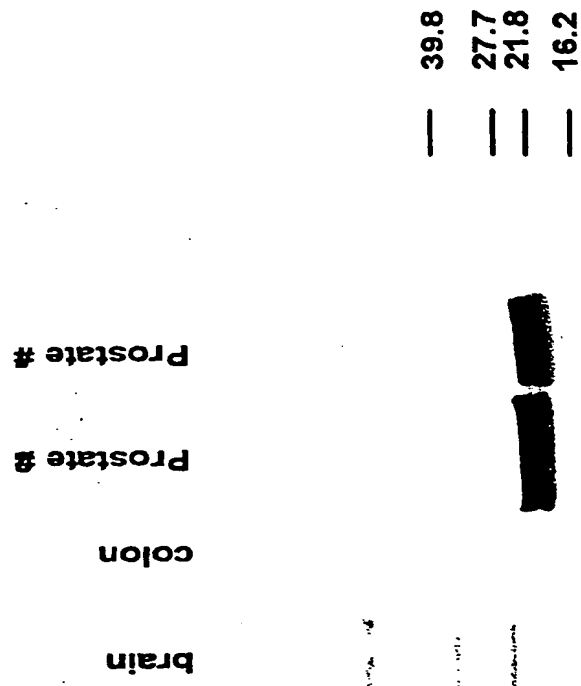


Fig. 13

## SEQUENCE LISTING

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&lt;210&gt; 6

&lt;211&gt; 1247

&lt;212&gt; DNA

&lt;213&gt; HUMAN

&lt;400&gt; 6

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&lt;210&gt; 7

&lt;211&gt; 104

&lt;212&gt; PRT

&lt;213&gt; HUMAN

&lt;400&gt; 7

Met Trp Val Pro Val Val Phe Leu Thr Leu Ser Val Thr Trp Ile Gly

1 5 10 15

Glu Arg Gly His Gly Trp Gly Asp Ala Gly Glu Gly Ala Ser Pro Asp

20 25 30

Cys Gln Ala Glu Ala Leu Ser Pro Pro Thr Gln His Pro Ser Pro Asp

35 40 45

Arg Glu Leu Gly Ser Phe Leu Ser Leu Pro Ala Pro Leu Gln Ala His

50 55 60

Thr Pro Ser Pro Ser Ile Leu Gln Gln Ser Ser Leu Pro His Gln Val

65 70 75 80

Pro Ala Pro Ser His Leu Pro Gln Asn Phe Leu Pro Ile Ala Gln Pro

85 90 95

Ala Pro Cys Ser Gln Leu Leu Tyr

100

&lt;210&gt; 8

&lt;211&gt; 218

&lt;212&gt; PRT

&lt;213&gt; HUMAN

&lt;400&gt; 8

Met Lys Asn Arg Gly Ser Tyr Pro Pro Pro Val Ser Val Ser Ser Trp

1 5 10 15

Ala Cys Leu Leu Cys Leu Cys Pro Leu Asp Glu Val Ser Met Ser Tyr

20 25 30

Arg Ala Trp Cys Ile Gln Gly Asp Leu Val Ile Ala Glu Gln Gln Val

35 40 45

Leu Ala Leu Pro Pro Leu Pro Gln Leu Trp Val Trp Glu Gly Val Val

50 55 60

Gln Pro Pro Ala Ala Trp Gly Gly Pro Trp Ser Ala Ser Gly Cys Gln

65 70 75 80

Gln Gly Arg Gly Gly Val Leu Gly Asn Glu Gly Phe Ile Gly Leu Leu

85 90 95

Gly Glu Ala Pro Gln Pro Gln Ala Tyr His Leu His Pro Glu Ser Cys

100 105 110

Val Thr Met Trp Val Pro Val Val Phe Leu Thr Leu Ser Val Thr Trp

115 120 125

Ile Gly Glu Arg Gly His Gly Trp Gly Asp Ala Gly Glu Gly Ala Ser

130 135 140

Pro Asp Cys Gln Ala Glu Ala Leu Ser Pro Pro Thr Gln His Pro Ser

145 150 155 160

Pro Asp Arg Glu Leu Gly Ser Phe Leu Ser Leu Pro Ala Pro Leu Gln

165

170

175

Ala His Thr Pro Ser Pro Ser Ile Leu Gln Gln Ser Ser Leu Pro His

180

185

190

Gln Val Pro Ala Pro Ser His Leu Pro Gln Asn Phe Leu Pro Ile Ala

195

200

205

Gln Pro Ala Pro Cys Ser Gln Leu Leu Tyr

210

215

&lt;210&gt; 9

&lt;211&gt; 218

&lt;212&gt; PRT

&lt;213&gt; HUMAN

&lt;400&gt; 9

Met Lys Asn Arg Gly Ser Tyr Pro Pro Pro Val Ser Val Ser Ser Trp

1

5

10

15

Ala Cys Leu Leu Cys Leu Cys Pro Leu Asp Glu Val Ser Met Ser Tyr

20

25

30

Arg Ala Trp Cys Ile Gln Gly Asp Leu Val Ile Ala Glu Gln Gln Val

35

40

45

Leu Ala Leu Pro Pro Leu Pro Gln Leu Trp Val Trp Glu Gly Val Val

50

55

60

65                      70                      75                      80

85                      90                      95

100                      105                      110

115                      120                      125

130                      135                      140

145                      150                      155                      160

165                      170                      175

180                      185                      190

195                      200                      205

210 215

<210> 10

<211> 183

<212> PRT

<213> HUMAN

<400> 10

Met Lys Asn Arg Gly Ser Tyr Pro Pro Pro Val Ser Val Ser Ser Trp  
1 5 10 15

Ala Cys Leu Leu Cys Leu Cys Pro Leu Asp Glu Val Ser Met Ser Tyr  
20 25 30

Arg Ala Trp Cys Ile Gln Gly Asp Leu Val Ile Ala Glu Gln Gln Val  
35 40 45

Leu Ala Leu Pro Pro Leu Pro Gln Leu Trp Val Trp Glu Gly Val Val  
50 55 60

Gln Pro Pro Ala Ala Trp Gly Gly Pro Trp Ser Ala Ser Gly Cys Gln  
65 70 75 80

Gln Gly Arg Gly Gly Val Leu Gly Asn Glu Gly Phe Ile Gly Leu Leu  
85 90 95

Gly Glu Ala Pro Gln Pro Gln Ala Tyr His Leu His Pro Glu Ser Cys  
100 105 110

Val Thr Met Trp Val Pro Val Val Phe Leu Thr Leu Ser Val Thr Trp



115

120

125

Ile Gly Glu Arg Gly His Gly Trp Gly Asp Ala Gly Glu Gly Ala Ser

130

135

140

Pro Asp Cys Gln Ala Glu Ala Leu Ser Pro Pro Thr Gln His Pro Ser

145

150

155

160

Pro Asp Arg Glu Leu Gly Ser Phe Leu Ser Leu Pro Ala Pro Leu Gln

165

170

175

Leu Pro Ala Pro Ser Cys Leu

180

&lt;210&gt; 11

&lt;211&gt; 375

&lt;212&gt; PRT

&lt;213&gt; HUMAN

&lt;400&gt; 11

Met Lys Asn Arg Gly Ser Tyr Pro Pro Pro Val Ser Val Ser Ser Trp

1

5

10

15

Ala Cys Leu Leu Cys Leu Cys Pro Leu Asp Glu Val Ser Met Ser Tyr

20

25

30

Arg Ala Trp Cys Ile Gln Gly Asp Leu Val Ile Ala Glu Gln Gln Val

35

40

45

Leu Ala Leu Pro Pro Leu Pro Gln Leu Trp Val Trp Glu Gly Val Val

50

55

60

Gln Pro Pro Ala Ala Trp Gly Gly Pro Trp Ser Ala Ser Gly Cys Gln

65

70

75

80

Gln Gly Arg Gly Gly Val Leu Gly Asn Glu Gly Phe Ile Gly Leu Leu

85

90

95

Gly Glu Ala Pro Gln Pro Gln Ala Tyr His Leu His Pro Glu Ser Cys

100

105

110

Val Thr Met Trp Val Pro Val Val Phe Leu Thr Leu Ser Val Thr Trp

115

120

125

Ile Gly Ala Ala Pro Leu Ile Leu Ser Arg Ile Val Gly Gly Trp Glu

130

135

140

Cys Glu Lys His Ser Gln Pro Trp Gln Val Leu Val Ala Ser Arg Gly

145

150

155

160

Arg Ala Val Cys Gly Gly Val Leu Val His Pro Gln Trp Val Leu Thr

165

170

175

Ala Ala His Cys Ile Arg Asn Lys Ser Val Ile Leu Leu Gly Arg His

180

185

190

Ser Leu Phe His Pro Glu Asp Thr Gly Gln Val Phe Gln Val Ser His

195

200

205

Ser Phe Pro His Pro Leu Tyr Asp Met Ser Leu Leu Lys Asn Arg Phe

210

215

220

Leu Arg Pro Gly Asp Asp Ser Ser His Asp Leu Met Leu Leu Arg Leu

225

230

235

240

Ser Glu Pro Ala Glu Leu Thr Asp Ala Val Lys Val Met Asp Leu Pro

245

250

255

Thr Gln Glu Pro Ala Leu Gly Thr Thr Cys Tyr Ala Ser Gly Trp Gly

260

265

270

Ser Ile Glu Pro Glu Glu Phe Leu Thr Pro Lys Lys Leu Gln Cys Val

275

280

285

Asp Leu His Val Ile Ser Asn Asp Val Cys Ala Gln Val His Pro Gln

290

295

300

Lys Val Thr Lys Phe Met Leu Cys Ala Gly Arg Trp Thr Gly Gly Lys

305

310

315

320

Ser Thr Cys Ser Gly Asp Ser Gly Gly Pro Leu Val Cys Asn Gly Val

325

330

335

Leu Gln Gly Ile Thr Ser Trp Gly Ser Glu Pro Cys Ala Leu Pro Glu

340

345

350

Arg Pro Ser Leu Tyr Thr Lys Val Val His Tyr Arg Lys Trp Ile Lys

355

360

365

Asp Thr Ile Val Ala Asn Pro

370

375

&lt;210&gt; 12

&lt;211&gt; 141

&lt;212&gt; PRT

&lt;213&gt; HUMAN

&lt;400&gt; 12

Met Trp Asp Leu Val Leu Ser Ile Ala Leu Ser Val Gly Cys Thr Gly

1

5

10

15

Glu Ile Gly Gly Ile Lys Glu Gly Gly Arg Val Leu Thr Leu Met Leu

20

25

30

Lys Pro Phe Ser Ser His Pro Val Pro Gln Pro Arg Pro Phe Ser Pro

35

40

45

Gln Phe Ser Pro Asp Asn Val Pro Leu Thr Leu Pro His Cys Asn Ser

50

55

60

Pro His Ala His Thr Arg Ser Pro Leu Pro Pro Thr Tyr Leu Arg Pro

65

70

75

80

Phe Ser Pro Leu Pro Ser Gln Ile Pro Ala Pro Ser Cys Phe Thr Lys

85

90

95

Glu Gln Val Pro Arg His Leu Cys Val Ser Leu Tyr Gly Val Gln Asn

100

105

110

Leu Ser Arg Thr Ser Leu His Ala Thr Gly Ser Leu Asp Pro Ile Thr

115

120

125

Gly Leu Pro Pro Glu Pro Leu Ser Pro Thr Thr Val Tyr

130

135

140

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